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THE EFFECT OF CARBON DIOXIDE ON THE GROWTH OF THE TUBERCLE BACILLUS.

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NUMEROUS researches on the effect of CO_2 on the growth of micro-organisms up to 1928 have been summarized by Valley (1928). This early work deals with bactericidal and inhibitory action, beneficial action and indispensability. The latter has been largely cleared up by the work of Gladstone, Fildes and Richardson (1935). These workers have shown that in the case of the ten organisms examined by them CO_2 is essential to their growth. They have also shown that bacteria differ in their susceptibilities to the removal of CO_2, a finding which is in agreement with the work of Valley and Rettger (1927). It now seems very probable that CO_2 is essential to the growth of most bacteria including the tubercle bacillus.

CO_2 has a beneficial effect on the growth of many organisms in concentrations up to 10 per cent.; the question of a possible inhibitory or even bactericidal action of CO_2 is by no means settled. Up to 1932 all the work in this field had been done on solid media, and it therefore suffered from all the drawbacks attendant on this use. In many cases no controls of any sort were done. A further criticism to much of this early work is that the cultures were incubated in a closed atmosphere which was often of comparatively small volume, no attention being paid to changes in the composition of this atmosphere brought about by the metabolism of the bacteria during growth. Cases of inhibition by CO_2 with strict aerobes, including the tubercle bacillus, may well have been due to a reduction in the O_2 content of the atmosphere and not to an increased CO_2 concentration.

Since 1932 good evidence has been obtained that certain concentrations of CO_2 do inhibit the growth of some bacteria and moulds; most of this work has been concerned with the preservation of meat and has dealt with organisms having a low temperature optimum. (Moran, Smith and Tomkins, 1932; Callow, 1932; Tomkins, 1932; Coyne, 1932, 1933; Haines, 1933). None of the above work dealt with the tubercle bacillus.

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None of the work on the tubercle bacillus can be regarded as conclusive. Corper, Gauss and Rensch (1921) stated that the human type of tubercle bacillus required some CO_2 for growth, but that 3 per cent. was inhibitory and 6 per cent. was bactericidal. These results were not confirmed in a later paper (Corper, Lurie and Uyei, 1927) in which it was stated that no inhibitory action of CO_2 was observed until the concentration had risen to 50 per cent.; there was considerable inhibition with 70 per cent. The technique was immensely better in the work described in the second paper, but complex solid media were still used, and no check was made on the pH. Results comparable to the later work of Corpers, Lurie and Uyei (1927) were also obtained by Novy and Soule (1925).

Recently Ebina, Nakamura and Inomata (1938) used a liquid synthetic medium of pH 6·6 in flasks which were exhausted and filled with the required gas mixtures, this operation being repeated at intervals of 2 to 3 days. The authors concluded that in the presence of 20 per cent. of O_2 the optimum concentration of CO_2 for the growth of the human and bovine types of tubercle bacillus is 5 per cent., and for the avian type is 0 to 10 per cent. Their results will be discussed more fully at the end of this paper.

In the experiments about to be described an attempt has been made to control as many variable factors as possible in order that the effect of CO_2 on the growth of the tubercle bacillus might be more clearly seen. The measures taken to secure this end were as follows:

(1) The organism was grown in an atmosphere which was maintained constant by a continuous flow of gas through the culture flask.

(2) The percentage of O_2 was maintained constant at about 20 per cent. in all cases; only the percentage of CO_2 was varied.

(3) A strain of organism was used which would grow readily on a liquid synthetic medium, thus enabling a medium of clearly-defined composition to be used.

(4) The maintenance of equilibrium between the gaseous and liquid phases was facilitated by bubbling the gas through the medium.

(5) Changes in volume of the cultures during incubation were prevented.

(6) The pH of the cultures was maintained at the same constant value by making use of the buffering action of the CO_2–$NaHCO_3$ system. This involved the use of varying amounts of $NaHCO_3$ in the different cultures, which therefore differed in their total salt contents. This variation in salt content introduced the only variable factor other than the concentration of CO_2. An attempt to control it was made by determining the effect on growth of concentrations of NaCl equivalent to the $NaHCO_3$ concentrations.

Only one strain of tubercle bacillus was used in the course of this work. It was the well-known human type strain H.37.

TECHNIQUE.

Apparatus.

The apparatus is shown diagrammatically in Fig. 1. A stream of air was passed at a constant speed through a 250 ml. bottle A. CO_2 and O_2 were also passed into A at such rates that the gas issuing from A contained about 21 per

cent. of O_2 and the desired percentage of CO_2. The arrangements used to maintain the rates of flow constant have not been included in Fig. 1, as they were somewhat complicated and not essential to the description of the experimental procedure. In brief, a constant pressure was maintained at each end X and Y of the apparatus by means of electrically operated pressure releases similar in principle to a thermo-regulator. Constant pressures of CO_2 and O_2 were maintained in gas holders by similar means, gas being automatically released into the gas holders from high-pressure cylinders fitted with Adam's reducing valves when the pressures of CO_2 and O_2 in these gas holders were higher than the pressure in the bottle A. The flow of each gas into A was regulated by means of an ordinary glass stopcock and a flow-meter. The gas mixture issuing from A then passed into the bottle B, containing distilled water maintained at 50° C. by immersion in a water bath. Here the gas took up water vapour and then passed through a tube C (12 in. by 1½ in.) packed with glass wool and maintained at a temperature of 110° C. in an oven. The issuing gas was then sterile and was led into the incubator where it first passed

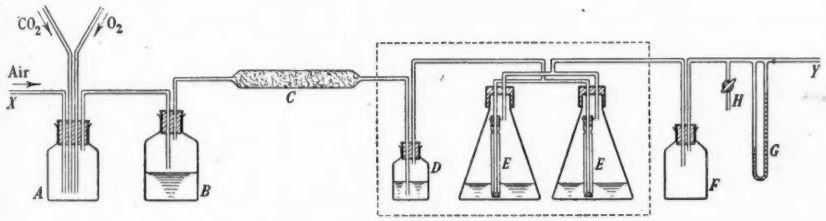

FIG. 1.—Diagram of the apparatus.

through the bottle D containing sterile distilled water, and then through the culture flasks E, E. These flasks were connected in parallel. If passage of the gas mixture through B had not resulted in sufficient water vapour being taken up to saturate it at 37° C., then more water was taken up from D; conversely, if too much water had been taken up in B, the excess was deposited in D. Thus the gas which reached the culture flasks was saturated with water vapour at 37° C. and changes in volume of the medium during incubation were largely prevented. The culture flasks are shown in detail in Fig. 2. They consisted of 500 ml. rimless Erlenmeyer flasks fitted with thick rubber caps through which passed the inlet tube for the bubbling device and an outlet tube. The bubbling device enabled the gas mixture to be bubbled through the medium without disturbing the surface and so breaking up the pellicle of tubercle bacilli that formed during incubation. This pellicle is easily caused to sink to the bottom of the medium, and when it does so growth ceases. From the culture flasks the gas passed through the bottle F immersed in cold water to remove excess water vapour from the cooled gas, and then through the flow-meter G, where its rate of flow was measured. Samples of gas were taken for analysis by means of the tap H inserted between the bottle F and the flow-meter. In the actual experiments five such pieces of apparatus

were run simultaneously, each with a different concentration of CO_2, and all the cultures were in the same incubator. The two flasks E, E contained duplicate cultures in every case.

Gas Mixtures.

The following gas mixtures were employed : O_2, 21 per cent. in all cases ; CO_2, nil, 2·5, 5·0, 10·0 and 20·0 per cent. These percentages were only approximate. The gas mixture called O_2, 21 per cent., CO_2, nil, was simply a stream of air.

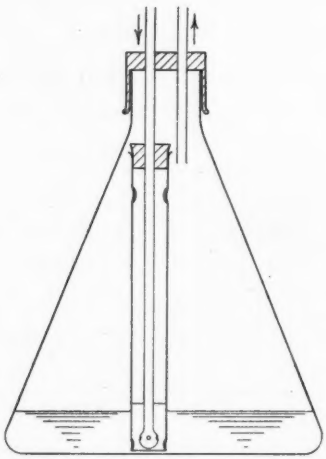

FIG. 2.—Culture flask.

Rate of Flow of Gas Mixtures.

The object of having a continuous flow of gas through the culture flasks was to maintain in them an atmosphere of constant composition. Since aerobic bacteria continually take up O_2 and give out CO_2 as a consequence of their metabolic activities, it was necessary to have a minimum rate of flow of each gas mixture in order to keep the composition of the gas in the culture flask at the required value. The minimum rate of flow for each gas mixture was calculated as follows :

Suppose the composition of the gas in the culture flask is to be x per cent. CO_2 and 21 per cent. O_2, and that a maximum variation of 10 per cent. in the concentration of each gas is permissible. Loebel, Shorr and Richardson (1933) found that tubercle bacilli (human strain H.37) respiring in Long's medium had an O_2 uptake of 1·0 to 2·7 μl. per mg. wet weight of bacilli per hour, which is about 20 μl. per mg. dry weight per hour, assuming the dry

weight of tubercle bacilli to be 10 per cent. of their wet weight. The respiratory quotient of the tubercle bacillus when metabolizing glycerol was found by Novy and Soule (1925) to be 0·85. Using, however, the value R.Q. = 1 to be on the safe side, the CO_2 output of tubercle bacilli growing on Long's medium is about 20 µl. per mg. dry weight per hour. The dry weight of tubercle bacilli harvested from 150 ml. of Long's medium after three weeks' incubation at 37° C. is about 0·5 g. This weight of bacteria therefore produces CO_2 at the approximate rate of 10 ml. per hour. If the gas mixture is flowing through the culture flask at V ml. per hour, this rate of CO_2 production will result in the percentage of CO_2 in the outflowing gas being $\frac{10}{V} \times 100$ per cent. higher than that in the inflowing gas. Now a 10 per cent. increase in the concentration of CO_2 in the gas mixture is $\frac{x}{10}$ per cent., and if the percentage of this gas is not to increase by more than this amount, the rate of flow of the gas mixture must not be less than that given by the equation $\frac{10}{V} \times 100 = \frac{x}{10}$, i.e. V must not be less than $\frac{10{,}000}{x}$ ml. per hour or $\frac{500}{3x}$ ml. per minute. The same formula will, of course, hold for a calculation of the minimum rate of flow based on a 10 per cent. variation in the O_2 content of the medium. For gas mixtures containing 2·5, 5·0, 10·0 and 20·0 per cent. CO_2 the minimum rates of flow per culture flask are 67, 33, 17 and 8·5 ml. per minute, based on the CO_2 content. Based on the O_2 content of the gas mixture the minimum rate of flow is 8 ml. per minute per flask. In the experiments the rates of flow were increased slightly to 100, 50, 25 and 12·5 ml. per minute per flask respectively. In the case of the culture incubated in a stream of air the rate of flow of the air was 200 ml. per minute per flask. The lower rates of flow were employed with the higher concentrations of CO_2.

Media.

Two stock solutions A and B were used. Solution A consisted of 0·15 *M* asparagine, 0·015 *M* $MgSO_4$, 0·0015 *M* ferrous ammonium sulphate, 0·03 *M* sodium citrate, 1·5 *M* glycerol, 0·075 *M* K_2HPO_4 and 0·02 *M* KH_2PO_4. The two phosphate salts are in such a proportion as to buffer the solution at pH 7·4. Solution B was a 0·75 *M* solution of $NaHCO_3$. It was prepared by saturating a 0·375 *M* solution of Na_2CO_3 with CO_2. Immediately prior to use it was sterilized by autoclaving and then resaturated with CO_2 from a cylinder, the gas being passed through a sterile cotton-wool filter before entering the solution.

The individual media were prepared by measuring 50 ml. of solution A into each culture flask together with (100–x) ml. of distilled water. The flasks were then sterilized in the autoclave and x ml. of sterile solution B were added with a sterile pipette. The value of x varied according to the percentage of CO_2 which was to be maintained in the gas phase and is nil for a CO_2-free atmosphere and 2·5, 5·0, 10·0 and 20·0 ml. for atmospheres containing 2·5,

5·0, 10·0 and 20·0 per cent. CO_2 respectively. The final media therefore had the following compositions :

	M conc.
Glycerol	0·5
Asparagine	0·05
$MgSO_4$	0·005
Ferrous ammonium sulphate	0·0005
Sodium citrate	0·01
K_2HPO_4	0·025
KH_2PO_4	0·007
$NaHCO_3$	$\frac{x}{200}$, where x = per cent. CO_2 in gas phase.

The medium containing no $NaHCO_3$ will be referred to as the basal medium.

All these media are buffered at pH 7·4 by the phosphate system, and those containing bicarbonate are also buffered at this pH by the bicarbonate-CO_2 system, for $\frac{x}{200}$ *M* $NaHCO_3$ at pH 7·4 and 37° C. is in equilibrium with a gas phase containing x per cent. CO_2. That this buffering was adequate will be seen from the pH values of the media after incubation which are given below.

It will be noted that the media used contained no NH_3 apart from the very small quantity added as ferrous ammonium sulphate. The reason for this was that NH_3 would probably have been carried off from the cultures during aeration and, owing to the different speeds of aeration in the different cultures, the loss would not have been the same in all cases. Preliminary experiments showed that the strain of tubercle bacillus employed would grow quite well without NH_3, although not so well as with it, and so ammonium salts were omitted from the media.

Sterilization of Apparatus, Sowing and Incubation.

The bottles D, about half full of distilled water, and all the connections between C and the culture flasks were sterilized by autoclaving and assembled just before commencing the experiment. The tubes C were autoclaved before being placed in position in the oven, but were not subsequently touched. Each flask of medium was sown with a 5 mm. loopful of the growth from a three-weeks-old culture of *M. tuberculosis* (human type strain H.37) on the basal medium. The rubber caps fitted with inlet and outlet tubes, and separately sterilized, were fitted to the flasks which were then connected up to the apparatus and the gas flow started. Incubation of the cultures was at 37° C.

Analysis of the Gas Mixtures.

The gas issuing from the culture flasks was analysed daily. It was drawn off through the tap H direct into the burette of a Haldane gas analysis

apparatus. The average compositions over the whole period of incubation, together with the standard deviations from the mean, were as follows :

	Per cent. CO_2.	Per cent. O_2.
(1)	$0{\cdot}24 \pm 0{\cdot}03$	$20{\cdot}3 \pm 0{\cdot}2$
(2)	$2{\cdot}58 \pm 0{\cdot}13$	$19{\cdot}9 \pm 0{\cdot}5$
(3)	$4{\cdot}86 \pm 0{\cdot}39$	$20{\cdot}0 \pm 0{\cdot}2$
(4)	$10{\cdot}3 \pm 0{\cdot}45$	$21{\cdot}1 \pm 0{\cdot}2$
(5)	$19{\cdot}7 \pm 2{\cdot}9$	$22{\cdot}5 \pm 1{\cdot}3$

With the exception of (5) the composition of the gas phase was within the maximum permissible variation of 10 per cent. (1) gives the composition of gas issuing, when the gas flowing into the culture flask was air ; initially the percentage of CO_2 was that of the laboratory air (about 0·1 per cent.), but it rose rapidly until after two days' incubation it was 0·21 per cent., and then slowly until the close of the experiment, when it was 0·27 per cent. The increase was probably due to the metabolism of the bacteria.

Harvesting and Estimation of Growth.

Incubation of the cultures was stopped after two weeks and the final pH of each culture was determined to 0·1 unit with bromthymol blue ; no alteration had taken place in any of the flasks. The cultures were then sterilized by autoclaving and the growth was filtered off and estimated by determining the total N built into bacterial substance. N estimations were carried out by the Kjeldahl method as described in a previous publication (Davies, 1939). The relationship between growth and CO_2 concentration is shown in Fig. 3. The values for growth are the mean of duplicate cultures which agreed to within 15 per cent. or less, usually less than 10 per cent. ; the largest variation occurred with the smallest growths. The final volumes of the media were also measured and they were all between 142 ml. and 167 ml. as compared with the initial volume of 150 ml.

Effect of Rate of Gas Flow.

It was thought that the better growth on the media incubated in the lower percentage of CO_2 might have been due to the higher rates of flow of gas through these cultures. An experiment was therefore started in which the gas flow was the same in all cases : viz. 100 ml. per minute per flask. Owing to unforeseen circumstances this experiment had to be terminated after only one week's incubation and the amount of growth was not estimated. There was, however, a sufficiently great divergence between the growth in 2·5 per cent. CO_2 and 20 per cent. CO_2 to indicate that the final results would have been substantially the same as before.

Effect of NaCl on Growth.

Although the results so far obtained indicated an optimum growth of this strain of tubercle bacillus at pH 7·4 in the presence of 2·5 per cent. CO_2 and 0·0125 *M* $NaHCO_3$, it could not be assumed that this effect was due solely

to the CO_2. In fact the effect could be ascribed to one or to a combination of three causes. Firstly, it could be due to the free CO_2 in the medium and in the gas phase; secondly, it could be due to $NaHCO_3$ acting specifically as a salt of H_2CO_3; thirdly, it could be due to $NaHCO_3$ acting simply through ionic effects which would be shown by any salt.

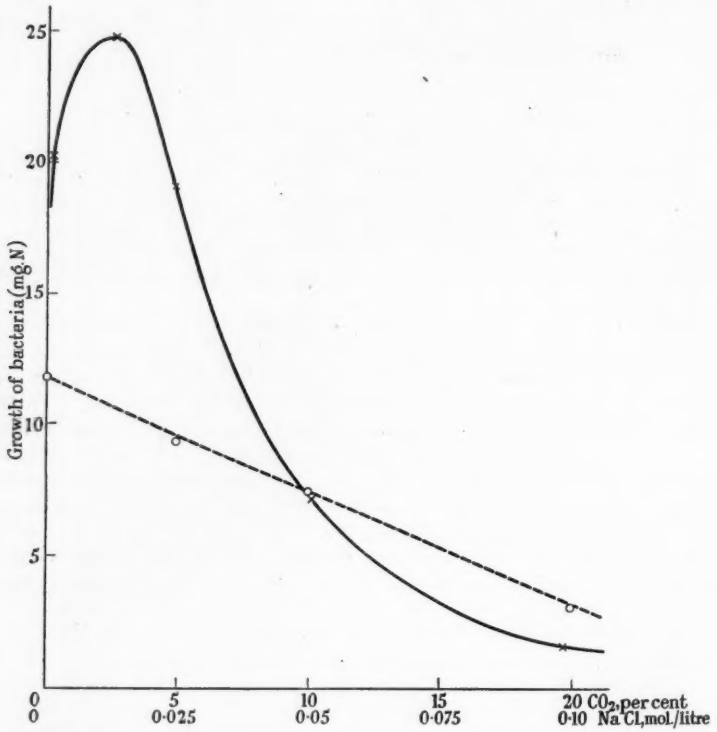

FIG. 3.—Effect of carbon dioxide and sodium chloride on the growth of tubercle bacillus (H. 37).
×——× = carbon dioxide.
⊙- - - -⊙ = sodium chloride.

No attempt was made to differentiate between the first two possibilities, but an attempt was made to rule out the last by investigating the effect of NaCl on the growth of the organism. The media were identical with those used previously except that the $NaHCO_3$ was replaced by an equimolar quantity of NaCl. These media were sown as before and incubated "stagnant," i.e. without having air bubbled through them. This may have introduced a source of error because growth on the basal medium was less under these conditions than it was when air was bubbled through it. Unforeseen circumstances prevented further experiments to decide this point. The growth was

harvested after two weeks' incubation and estimated as before. The relationship between growth and NaCl concentration is shown in Fig. 3. The pH of each culture was also estimated and was found to be between 6·9 and 7·4. Stagnant cultures were also set up in which the media were identical with those used in the experiments with CO_2. These cultures failed to grow, and on determining the pH of each medium after two weeks' incubation it was found in every case to be over 9·0. The alkalinity was obviously due to loss of CO_2 by diffusion from the $NaHCO_3$ in the medium.

DISCUSSION.

The results that have been obtained in these experiments show that at pH 7·4 there exists an equilibrium mixture of CO_2 and $NaHCO_3$ which gives an optimum growth of the human-type tubercle bacillus, strain H.37. This occurs with 2·5 per cent. of CO_2 in equilibrium with 0·0125 *M* $NaHCO_3$. With CO_2 and $NaHCO_3$ concentrations greater or less than this, the amount of growth is diminished.

In most of the work on this subject attention has been devoted entirely to the concentration of CO_2 in the gaseous phase, the concentration in the medium being disregarded. There is no proof that, even with solid media, gaseous CO_2 is the active agent; the effect may equally well be due to the CO_2 dissolved in the medium. With complex solid media such as have been used in most of the experiments with the tubercle bacillus it is difficult to know the concentration of CO_2 in the medium, and it is therefore difficult to compare the results described here with those of other workers, except Ebina *et al.* (1938). These workers employed a liquid synthetic medium having an initial pH of 6·6, and although they did not state the values of pH attained under the influence of high concentrations of CO_2, it can be calculated that with 20 per cent. of CO_2 the pH of their medium would not fall below 6·4. It is probable, therefore, that for CO_2 concentrations of 20 per cent. or less, their results do not include any appreciable disturbing effects due to change in pH. They state that 5 per cent. is the optimum concentration of CO_2 for growth of the human type of tubercle bacillus. If, however, their values for the amount of growth are plotted against concentration of CO_2 the curves shown in Fig. 4 are obtained. These curves show that the optimum CO_2 concentrations for the three strains of human tubercle bacillus is probably less than 5 per cent., possibly about 2·5 per cent., in agreement with the results given in this paper.

Since the method employed in the present experiments for buffering the media involved the use of rather large concentrations of $NaHCO_3$ with the higher concentrations of CO_2, it was necessary to show that the observed effects were not simply salt effects. Fig. 3 shows the effect of adding NaCl to the basal medium. NaCl was chosen for these experiments because neither sodium nor chloride ions seem to be essential for the growth of the tubercle bacillus, and any effect of this salt on growth may therefore be considered to be a simple ionic effect. It is obvious from the curves of Fig. 3 that the responses in the two cases are quite different, and that the stimulating effect

of concentrations of CO_2 below 2·5 per cent., and the inhibitory effect of concentrations above 2·5 per cent. are, in fact, due to a specific action of CO_2. The experiments of Becker (1936) are of interest in this connection. Becker studied the effect of various acids on certain heterotrophic cells (not bacteria) and he found that CO_2 stood out quite clearly from the rest in its action on the cells; it was able to penetrate very rapidly into the cell and in high concentrations (0·03 *M*) had a narcotizing action. This property was not possessed by any of the other acids studied, and it had nothing to do with pH.

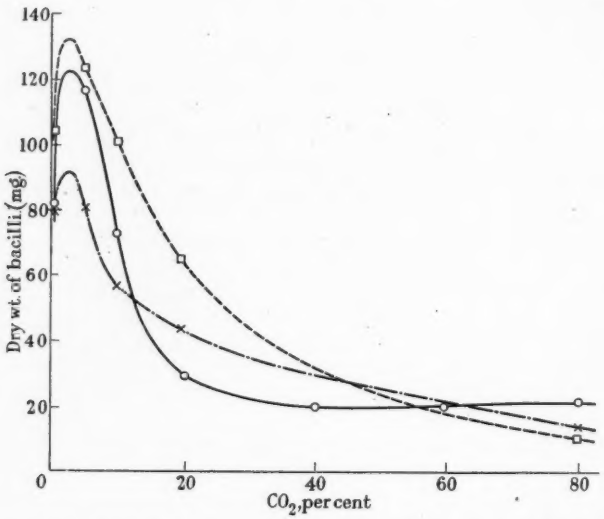

FIG. 4.—Curves derived from the findings of Ebina *et al.* (1938) for three strains of human-type tubercle bacilli.
⊙———⊙ = strain "Sakaikbara."
×—·—·—× = strain "Yamaguchi."
□– – –□ = strain "Sasaki."

The results obtained in these experiments would seem to have some significance with regard to the development of tubercle bacilli in the human lung. The normal concentration of CO_2 in the alveolar air of a person at rest and lying down is about 5·5 per cent. This value is well above the optimum concentration for the growth of the tubercle bacillus, and therefore the conditions in the normal lung are not the most favourable for the multiplication of this organism. If, through any cause, the concentration of CO_2 is diminished, the conditions become more favourable to the growth of the tubercle bacillus; if the concentration is increased, conditions become still less favourable to growth. Exercise considerably reduces the CO_2 concentration in the alveolar air, and even when standing still the CO_2 concentration is lower than when lying down (4·5 as against 5·5 per cent.). Absolute rest in bed has long been recognized as one of the best treatments for early pulmonary tuberculosis,

and it seems that the value of this treatment may be partly correlated with the effect on the concentration of CO_2 in the alveolar air. Immobilization of the whole or part of a lung will also produce an increase in the concentration of CO_2 in the immediate vicinity of the immobilized part, since the gases surrounding it will acquire the composition of stagnant tissue gases; these contain about 6–6·5 per cent. of CO_2 (Campbell, 1931). The effectiveness of lung collapse as a treatment for pulmonary tuberculosis may also then be partly correlated with the local increase in CO_2 concentration which it produces.

SUMMARY.

(1) The effect of carbon dioxide on the growth of the human-type tubercle bacillus strain H.37 has been investigated under strictly defined conditions.

(2) Optimum growth of this organism under the conditions used was obtained in the presence of 2·5 per cent. of carbon dioxide in equilibrium with 0·0125 *M* sodium bicarbonate at pH 7·4.

(3) The effect on growth is due to a specific action of carbon dioxide and not to the presence of a particular concentration of ions in the medium.

(4) A possible relationship between the concentration of carbon dioxide in the lungs, and the effectiveness of certain methods of treating pulmonary tuberculosis is discussed.

I wish to express my thanks to Dr. S. R. Gloyne for his continual encouragement and advice throughout this research, and to Dr. M. Stephenson for much helpful advice.

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THE OUTPUT OF LYMPHOCYTES FROM THE THORACIC DUCT IN CATS AND RABBITS.

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IN spite of the wide divergence of views on the precise origin, relationship and function of the lymphocyte it remains a fact that a large number of them are produced daily in the lymph glands. That many of the circulating lymphocytes reach the blood stream by way of the thoracic duct has long been recognized, but it is only comparatively recently that any simultaneous estimations of the lymphocytes in the duct lymph and the blood have been made. (One experiment by Biedl and Decastello (1901) is the only exception.)

Heidenhain (1891) measured the rate of flow from the duct in the dog under various conditions, but did not estimate the numbers of lymphocytes in his samples.

Winternitz (1895) also used dogs and found 2000–7000 lymphocytes per c.mm. to be the normal figure for fasting animals (with one exception of 22,000). He found the rate of flow to be equivalent to 9·6 c.c. per hour in one animal.

Biedl and Decastello (1901) calculated from Winternitz's figures for cell counts and their own measurements of rate of flow that in a 10 kg. dog about 10^9 lymphocytes would enter the blood stream daily from the duct. In such an animal the circulating lymphocytes would number about $1{\cdot}6 \times 10^9$. In their experiments they either opened or ligated the thoracic duct and often the right duct as well. In either case they found a drop in the blood lymphocytes of about 60 to 70 per cent. accompanied by a rise in the polymorphs up to 100 per cent. After a time the lymphocyte values returned to normal due to the opening up of collateral lymph pathways.

Selinoff (1904) made thoracic duct fistulae in dogs and found a rapid drop in the blood lymphocytes, a rise in the polymorphs, and no change in the red cells. The dogs took about three days to recover from the effects of the operation and even then were very somnolent as long as the lymph was flowing.

Crescenzi (see Banti, 1904) observed a fall in the blood lymphocytes in dogs with thoracic duct fistulae.

Rous (1908*a*) showed that much care was needed in interpreting the figures of earlier workers because muscular movements such as struggles produced a marked increase, not only in the rate of flow, but also in the total lymphocyte output. This increase was partly compensated for by a subsequent decrease. His experiments on quiescent dogs gave fairly steady rates of flow and cell

counts. Florey (1927) showed that slight pressure on a lymph gland was enough to double the cell count in the efferent lymphatics. Farris' (1938) recent experiments on rats also showed that the lymphocytes in the blood rose very considerably after a short struggle.

Bunting and Huston (1921) were apparently the first to use rabbits. They tied the duct and noted a large drop in the blood lymphocytes. In the duct, just after death, they found 20,000 to 30,000 lymphocytes per c.mm. They were not successful in cannulating the duct except with very fine cannulae, in which clotting soon occurred. (A technique for overcoming this difficulty will be described later.) More recently Yoffey (1932, 1933 and 1936) repeated these experiments on dogs. He counted the lymphocytes in the blood and in the lymph from the cannulated thoracic duct and also measured the rate of flow in the same animal. He thus showed that the daily inflow of lymphocytes into the blood stream via the duct was from two to two and a half times the number of lymphocytes normally present in the circulation. He also pointed out that this calculation gave a minimum figure as not all the lymphocytes enter the blood by this route. Some enter the veins on the right side of the neck and probably some directly into the venous system in the lymph glands. Most writers assert that the majority reach the blood via the duct, but Bloom (1938) thinks that the converse is true. In any case the replacement factor for the blood lymphocytes worked out from the rate of inflow via the duct will be too low and the life of any given lymphocyte in the blood stream will be too long. All one can say is that the blood lymphocytes are replaced at least so many times a day and that they circulate for not more than so many hours. In making this calculation one assumes that the lymphocytes that enter the blood from the thoracic duct are freshly made cells and do not return to the lymph from the blood in the lymphoid tissue. Such a circulation of lymphocytes had been thought on insufficient evidence to exist by some workers (Ehrlich, 1891 ; Sjövall, 1936).

The evidence for the direct entry of the lymphocytes into the blood stream in the lymphoid tissue is mainly histological. Renaut (1881) seems to have been the first to point out that certain minute blood vessels in the lymph glands have a curious type of cylindrical endothelium quite unlike the usual flattened cells but more resembling the epithelium of a duct. Thomé (1898) also described these anomalous vessels in lymphoid tissue and v. Schumacher (1899) saw lymphocytes pushing their way between these cells either to enter the blood stream or to leave it. To settle the question he counted the white cells in the afferent and efferent blood vessels of eight lymph glands from seven dogs and one rabbit. He stated that he found the counts higher in the veins but he gave no figures to support this statement. He does not appear to have done differential counts or red-cell counts to see whether there was any concentration of the blood from the removal of the fluid to the lymph, which is at least a theoretical possibility. If his observations were accurate they dispose of the argument that there is a circulation of lymphocytes. Zimmerman (1923) discussed the question and concluded that the evidence was in favour of the hypothesis that lymphocytes can and do enter the blood stream directly in the lymphoid tissue. Bloom (1938) went so far as to say that "most of the lymphocytes reach the general circulation by way of the blood

stream, although some of them are carried off in the lymph, and eventually reach the blood stream in this manner."

Whichever view be correct it seemed probable that further light on the general problem would be shed by a series of experiments on the output of lymph and lymphocytes from the thoracic duct.

EXPERIMENTAL.

Method of Collecting Lymphocytes.

Thoracic duct lymph contains a very high proportion of small lymphocytes. By using supra-vital staining for films the average number of large lymphocytes and monocytes was found in the cat to be about 5 per cent. of the total cells ; the same figure has been found for the rabbit and for the dog by Rous (1908*b*). With a good preparation it was possible to obtain lymph from the rabbit free from red blood cells. In the cat, however, it was found that, even with the cannula tied into the duct itself, there were often a few red cells present. This contamination was not sufficiently great to introduce many polymorphonuclear leucocytes into the lymph and there were never more than one or two seen in any count of 300 cells.

The amounts of lymph obtained by this method were not very large, especially in the cat, though a better yield was obtained from the rabbit, which also tended to have a greater number of cells per c.mm.

Operative Technique.

Thirteen cats, male and female, all about 3 kg. in weight were successfully operated upon. Three cats were anaesthetized with nembutal and ether, but the remainder were given ether during the dissection and after the cannula had been tied in they were decerebrated.

As a preliminary step the trachea was cannulated and loose ligatures placed round the carotids. A separate lateral incision was made over the external jugular vein and this was dissected down to its junction with the subclavian vein under the clavotrapezius muscle ; the muscle was then divided with a cautery and the venous junction exposed. The veins were freed by careful dissection, keeping to the outer side of the jugular vein as much as possible. This minimized the danger of letting air into the mediastinum, which previously had been found to be responsible for the death of several animals during the operation.

When the veins had been freed as much as possible the glistening white thoracic duct could be seen in the inner side of the jugular vein, whence it might take several courses. It might enter either the jugular or the subclavian vein or at their junction ; it might remain single or enter by two or more openings. The diagrams in Fig. 1 show three examples of the way the thoracic duct of the cat may terminate. In two-thirds of the animals the duct was large enough and sufficiently accessible to enable the cannula to be placed directly into it ; while in the others it was necessary to tie off the jugular and subclavian veins and all their tributaries, however small, and finally to place

a ligature round the innominate vein. The venous junction then filled with lymph and this could be removed by a cannula tied in the lower end of the

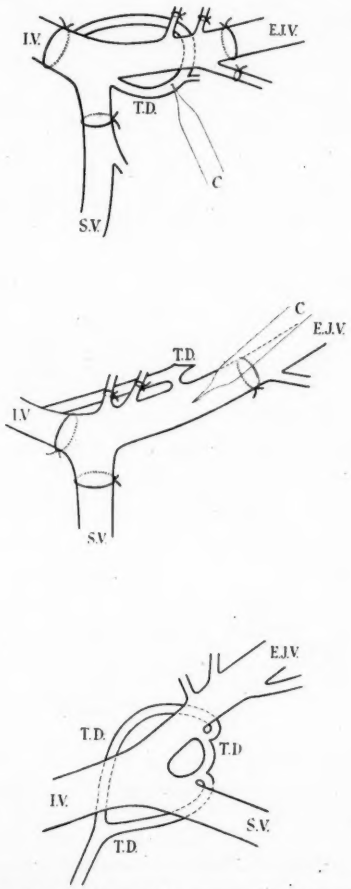

FIG. 1.—Diagrams showing the anatomy of the upper end of the thoracic duct in the cat.
Top: Entry into the junction of the jugular and innominate veins.
Middle: Entry into the jugular vein.
Bottom: Showing the thoracic duct dividing and entering the veins at two places.
E.J.V. = External jugular vein. S.V. = Subclavian vein. I.V. = Innominate vein.
T.D. Thoracic duct. C. = Cannula.

jugular. Much of the dissection was done with the help of a binocular dissecting microscope. With the cannula in place the cat was turned over and decerebrated. A few minutes after the decerebration the cat was turned on its back once more and the collection of the lymph begun. A piece of

rubber tubing was connected to the cannula and the lymph allowed to flow steadily into a cylinder. In order to prevent the lymph from clotting 3 per cent. sodium citrate was introduced through a needle inserted in the rubber tubing and running down to the base of the cannula. The citrate flowed at the rate of 1 c.c. an hour from a special apparatus similar in principle to the burette described by Burn and Dale (1924). The method of indirect cannulation of the thoracic duct was first described by Gaertner and Roemer (1892).

Cell counts were done at intervals on the pure lymph and in order to do this the rubber tubing was removed, the cannula emptied and allowed to refill with fresh lymph.

A complete blood count was done when the collection of the lymph was started and at intervals of four hours thereafter.

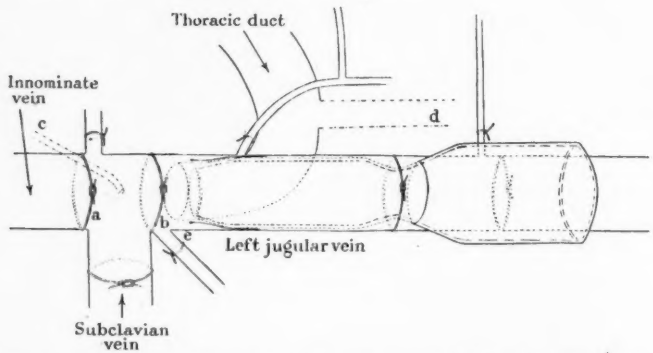

FIG. 2.—Diagram of the thoracic duct and left jugular vein in the rabbit. The left cervical duct may join the thoracic duct as at " d ", or it may enter the vein separately as at " e." " a " and " b " show alternative positions for the lower ligature. The small vein " c " is inconstant. A cannula is shown in position.

For the rabbits a similar technique was employed. The animal was anaesthetized by intravenous nembutal supplemented by ether and the anaesthetic maintained by further subcutaneous injections of nembutal. The trachea was sometimes cannulated as the first step for by doing so a clear airway was obtained and this diminished the respiratory movements at the base of the neck. The jugular vein was exposed and followed down to its junction with the subclavian vein. The greatest care was taken during the dissection to prevent any haemorrhage into the tissues as this obscured the very small tributary veins that had to be tied. The thoracic duct in this animal follows a regular course and always enters the jugular vein on its inner border just above the entry of the subclavian vein.

The main veins and all the tiny tributary veins were then tied off. Finally the innominate vein was ligatured and the lymph removed by a cannula in the lower end of the jugular vein (Fig. 2).

Twenty-one rabbits were successfully operated upon in this way and the lymph collected as in the cats.

The Production of Lymphocytes in the Cat.

In all the animals the lymph flowed steadily through the cannula for several hours, but in those animals kept alive for more than 10 hours the output of lymph became progressively smaller and finally ceased; even so the animals still appeared to be in good condition. At this point, however, several c.c. more could always be obtained by massaging the abdomen.

Table I gives a summary of the results in 10 cats kept alive for 6 hours or more. The figures given refer to the first 6 hours of the experiment. The starved cats had received no food for the preceding 24 hours and those described as "fed" had been given fish or meat about one hour before the operation was begun. The figures given for the cell counts of the lymph are the average for at least three counts made at different times.

From the table can be seen the well-marked difference between the amount of lymph flowing in the fed and the starved cats; while, on the other hand, the number of lymphocytes per c.mm. is a very variable figure and not dependent on whether the animal was fed or not. From the last column it can be seen that enormous numbers of lymphocytes must enter the circulation every 24 hours.

The total number of lymphocytes in the whole circulation can be roughly calculated from the figures of the blood count and taking the blood volume of the cat to be 6·5 per cent. of its weight (Welcker, 1925).

TABLE I.—*Production of Lymphocytes in the Cat.*

Cat No.	Condition.	Total lymph flow for first 6 hrs. (c.c.).	Average per hr. (c.c.).	Lymphocytes per c.mm. (average of 3 counts) ($\times 10^3$).	Total number lymphocytes put out in 6 hours ($\times 10^6$).
17	Starved	6·0	1·0	6·8	40·8
20	,,	8·4	1·4	5·9	50·1
23	,,	6·0	1·0	16·3	97·8
24	,,	7·2	1·2	20·7	149
12	Fed	29·0	4·9	28·3	821
16	,,	31·1	5·2	13·5	420
18	,,	24·2	4·0	10·8	261
21	,,	26·2	4·4	6·0	157
22	,,	18·1	3·0	10·9	197
25	,,	26·7	4·4	16·1	430

Table II gives the figures for the number of lymphocytes per c.mm. of blood in six normal cats. The figures given are the average of those found in six successive counts on each animal. The average weight of these cats was 3·5 kg., and this gives a total of 1250 million lymphocytes present in the circulating blood.

From Table I it can be calculated that in the fed cats the number of lymphocytes entering the circulation in 24 hours from the thoracic duct alone would have varied from 620 to 3280 million. That is, the blood lymphocytes would have been replaced from one half to almost three times every 24 hours.

TABLE II.—*Lymphocytes in Normal Cat Blood.*

Cat No.	Lymphocytes per c.mm. blood.		
A3	5100	Sigma ±	600
A4	6040	,,	440
A5	4400	,,	300
A6	4980	,,	430
A7	5950	,,	900
A8	7000	,,	1200
Mean	5580	,,	850

The Production of Lymphocytes in the Rabbit.

The protocol of one experiment only is given; the results of the others are summarized in tabular form.

Rabbit, A30.—Weight 2·7 kg. Blood volume 125 c.c. (from formula of Dreyer, Ray and Walker, 1912). Blood lymphocytes before operation 6000 per c.mm. (average of 3 counts). Blood lymphocytes after dissection but at the beginning of the timed flow of lymph 2881 per c.mm. (see below). Dissection took 1½ hours. The lymph was collected for the next four hours, being measured in volume every ¾ hour. A cell count was done on freely-flowing lymph in the middle of each such period (Table III).

TABLE III.—*Lymphocyte Production by a Rabbit.*

Periods of ¾ hr.	Vol. of lymph (c.c.).	Lymphocytes c.mm. ($\times 10^3$).	Total lymphocytes in period ($\times 10^6$).
1st	4·3	13·60	58·480
2nd	3·0	66·80	200·400
3rd	1·6	8·47	13·552
4th	1·7	10·32	17·544
5th	1·9	26·22	49·818
6th	2·7	19·27	52·029
Total of 4½ hours	15·2	(24·11)	391·823

The above experiment and eight others like it are summarized in Table IV:

TABLE IV.—*Lymphocyte Production in Rabbits.*

Rabbit.	Weight (kg.).	Average lymph flow (c.c./hr.).	Total daily output of lymphocytes ($\times 10^9$).	Notes.
A30	2·7	3·4	2·09	Fed.
L6	2·5	4·5	5·90	,,
L9	2·2	9·0	6·69	,,
L10	2·15	4·4	5·54	,,
L15	2·5	3·2	3·00	Starved.
L17	2·5	5·2	3·34	Excess cabbage.
L18	2·46	3·1	4·91	Starved.
L19	2·5	7·3	3·04	Excess cabbage.
L22	3·2	1·5	1·47	Starved.

Average = 4·00

Six more experiments are shown in Table V, the cell counts in these animals were done less frequently so that the figures are not so reliable.

TABLE V.—*Lymphocyte Production in Rabbits.*

No. of rabbit.	Weight (kg.).	Average lymph flow (c.c./hr.).	Total daily output of lymphocytes ($\times 10^9$).	Notes.
B2	1·9	6·4	3·02	Fed.
B3	2·6	5·3	2·96	,,
B5	2·7	3·8	2·20	,,
B8	2·6	6·5	1·97	,,
B9	..	5·3	3·18	,,
B17	1·6	10·1	2·84	,,

Average = 2·70

From 40 blood counts on 20 normal rabbits from the same stock the average blood lymphocytes had been found to be 7160 ($\sigma = 1600$) per c.mm. The blood volume ranged from 105 to 120 c.c., giving the total number of circulating lymphocytes of about 8×10^8. From Table IV, which is more reliable than Table V, the average daily output via the duct is some 4×10^9, which means that the lymphocyte population of the blood is replaced with fresh cells about five times a day. This is rather higher than that found above for cats (0·5 to 3·0) and by Yoffey (1932, 1933) for dogs (2·5).

These figures are certainly too low for all the animals were completely at rest throughout the experiment and exercise is known to increase very considerably not only the volume, but also the cellular content of the lymph (Drinker and Field, 1933 ; Rous, 1908*a*).

It then appeared of interest to try to find out to what extent the number of lymphocytes in the blood were maintained by those entering from the thoracic duct. This would be possible if any relationship could be found between the number of lymphocytes known to have been diverted in the lymph that had been collected and the fall in the blood lymphocytes that occurred during the same period.

Unfortunately this proved quite impossible in both cat and rabbit, the blood pictures being unstable and considerably altered by operative procedures.

For instance, five out of the thirteen cats whose thoracic ducts were cannulated showed a blood lymphocyte count of 2000 or less per c.mm., just after the cannula had been tied in place, compared with a normal count of 5000 per c.mm. (Table II). From Table VI can be seen the changes in the blood lymphocytes that occurred in four cats undergoing operations unconnected with the lymphatic system.

Thus, although in nearly all the cats observed with their thoracic ducts cannulated a fall in the blood lymphocytes occurred, the size of the fall was not proportional to the number of lymphocytes escaping in the lymph ; further it was no greater than that to be expected after an entirely different operation.

TABLE VI.—*Changes in the Number of Lymphocytes in the Blood of Cats undergoing Prolonged Operations.*

Cat. No.	Lymphocytes per c.mm. blood.		Procedure undertaken on the animals.
	Before operation.	6 hrs. later.	
76	7200	810	Decerebration after exposure of the colon.
77	6600	1100	Ditto.
85	5100	2200	Decapitation and collection of intestinal secretions.
86	6300	4800	Ditto.

In Table VII are summarized some of the figures actually obtained from these animals.

TABLE VII.—*Fall in Blood Lymphocytes Compared with Number Removed from Thoracic Duct.*

Cat. No.	Total number of lymphocytes from thoracic duct ($\times 10^6$).	Fall in blood lymphocytes per c.mm.	Calculated total fall of the number of lymphocytes in the blood ($\times 10^6$).
1	100	+500	+97
5	56	3700	670
10	156	1300	250
12	980	4400	814
18	517	+200	+39
21	318	5000	975

Similar results were obtained with the rabbits.

It can be seen from these figures that the fall in the lymphocytes in the blood bears no relation to the numbers lost in the escaping thoracic lymph; the fact that in two of the animals the blood lymphocytes actually rose suggests that the place of those cells that should have entered from the thoracic duct may readily be taken by lymphocytes from other sources (Zimmerman, 1923).

There is a further reason for believing that the numbers of lymphocytes circulating in the blood are not maintained only by those lymphocytes entering the blood from the thoracic duct.

It can be seen from Table II that the blood lymphocytes of the normal cat varied only from 4700 to 7100 per c.mm. On the other hand the number of lymphocytes in the thoracic lymph in six successive cats varied from 5000 to 28,000 per c.mm.

A similar variation has been found in the lymph collected from the forelimb of rabbits by Menkin and Freund (1929). If, therefore, the level of the lymphocytes in the blood depended mainly upon the number entering by the thoracic duct one would expect to find it to be a much more variable figure.

These findings certainly lend support to the idea that the daily utilization of the lymphocytes must be even greater than that calculated solely from the numbers of cells entering the blood by the thoracic duct.

SUMMARY.

(1) Methods are described for collecting lymph continuously for several hours from the thoracic ducts of cats and rabbits.

(2) This lymph contains about 5 per cent. large lymphocytes and monocytes and the rest of the cells are small lymphocytes.

(3) The daily replacement factor for blood lymphocytes varies from 0·5 to 3·5 in the cat, to about 5 in the rabbit. Reasons are given for regarding this as a minimum figure.

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THE ENZYMES OF LYMPHOCYTES AND POLYMORPHONUCLEAR LEUCOCYTES.

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ENZYMES IN LYMPHOCYTES.

THERE are several papers in the literature describing the presence of enzymes in the lymphocytes, but the results are by no means uniform. This may be explained by the diversity of the sources, and the obvious impurity of some of the cell preparations utilized. Hitherto lymph glands, tuberculous pus and the white cells from the blood of cases of lymphatic leukaemia have been used. Fukuchi (1936, 1937) obtained lymphocytes in sufficient numbers from the leg lymphatics of rabbits to test for the presence of several enzymes.

A weak protease acting only in acid media was described by Opie (1905, 1906) (lymph glands), Hedin (1923) (lymph glands), Rona and Kleinemann (1931) (lymph glands), and Kleinemann and Scharr (1932) (pus from a turpentine abscess in a horse). An erepsin and trypsin were found by Hedin (1923) (lymph glands), Rona and Kleinemann (1931) (lymph glands), and Oelkers (1931) (leukaemic blood). Fukuchi (1936) (lymph), on the other hand, failed to confirm this. Reding (1937) (lymph glands) stated that lymphocytes contained a nuclease, and Morris and Boggs (1911) (leukaemic blood) found an amylase. Jochmann and Müller (1906) (tuberculous pus), Longcope and Donnhauser (1908) (leukaemic blood) and Dudgeon and Bond (1934) (tuberculous pus) believed that the lymphocytes contained no proteolytic enzymes.

A lipase was detected by Fiessenger and Marie (1909) (lymph glands), Bergel (1909) (tuberculous pus) and Fukuchi (1936) (lymph), but not by Nees (1921) (tuberculous pus). Bergel (1910, 1924) stated that the possession of this lipase was the key to the problem of the function of the lymphocytes. He believed that the metabolism of the body fat was mainly carried out by these cells. He has been severely criticized by Aschoff and Kamiya (1922, 1923), who stated that the cells called lymphocytes by Bergel were in fact histiocytes.

Ohyama (1924) studied the enzymes in extracts of lymph glands, and commented that the amylase, lipase and protease found present were in extremely small amounts. Furthermore he stated that the amount of lipase present in extracts of lymph glands was no greater than that present in extracts of other organs.

Methods.

The lymphocytes were obtained from the thoracic duct lymph of 13 cats and 12 rabbits. The amount supplied by the latter was much greater than from the former. The 13 cats only provided 270 c.c. of lymph although it was

collected over a period of 123 hours ; whereas the rabbits produced over 570 c.c. of lymph in a total collecting time of only 85 hours. Consequently the majority of the experiments were done on the cells of the rabbits, but enough material from the cats was available to confirm the results obtained with the rabbit lymphocytes.

The lymphocytes were collected from the lymph by centrifuging it at intervals as it flowed, and washing the cells twice with saline. This second washing of saline was tested for those enzymes found in the cells in order to see that none of the reactions observed were due to enzymes carried over from the lymph itself. The tests were uniformly negative.

The cells were then either stored at 0° C., or dried rapidly in an evacuated desiccator. Before use a 1 per cent. extract of lymphocytes was made by grinding them in 50 per cent. glycerol, and allowed to stand for 2 hrs. in order to extract the enzymes.

For proteolytic enzymes the method used was that described by Anson (1936, 1938), using denatured haemoglobin as a substrate, and testing for the split products in the form of tyrosine by the reduction of Folin's phenol reagent after the precipitation of the proteins with trichloracetic acid. The quantities used were one-tenth of those given by Anson, and the mixtures were incubated for 24 hrs. at 37° C., under toluol.

To test for erepsin a 5 per cent. solution of glycyl-glycine was used, and the amount of splitting estimated by the nitrogen liberated according to the method described by Van Slyke (1929).

The nuclease was estimated by measuring the amount of free phosphate liberated from a 1 per cent. solution of thymo-nucleic acid, using the method of Berenblum and Chain (1938). The quantities used were 0·5 c.c. of substrate with 0·5 c.c. of buffer and 0·2 c.c. of glycerol extract of the cells. The mixture was incubated at 37° C. for 24 hrs.

For the amylase the substrate was a 1 per cent. solution of starch at pH 6·2 and the increase in reducing sugars was estimated by the method of Miller and Van Slyke (1936).

The lipase was tested for by the method of Rona and Michaelis (1911), whereby a saturated solution of tributyrin in water is incubated with the cell extract and the amount of splitting determined by the change in the surface tension of the solution. This is measured with a stalagmometer which can readily be calibrated with solutions of tributyrin of known strength. The method has the advantage that solutions can be adequately buffered.

Results.

The amount of material available was small, but there was sufficient to enable the tests to be repeated, and the findings were consistent.

Protease.

A slight splitting of haemoglobin occurred between Hp 3·5–4·0 in all the tests, but there was none in the pH range of pepsin or trypsin. The enzyme therefore is probably one of the cathepsins, such as are found in most cells.

Erepsin.

No erepsin was detected in any of the tests.

Nuclease.

About 5 per cent. of the nucleoprotein solution was split at pH 4·6, but none in the alkaline range, and similarly a weak phosphatase was found using glycerophosphate as substrate.

Amylase.

There was a weak amylase present ; enough to split 10 per cent. of the starch solution in 24 hrs.

Lipase.

The test for lipase was always positive, and there was a complete splitting of a watery solution of tributyrin in 4 hrs. at 37° C.

It was found that an extract of fresh liver was able to split the tributyrin solution completely in a few minutes at room temperature. This means that the amount contained in the lymphocytes is probably comparatively small.

These findings, therefore, confirm the results of others and show that the lymphocytes contain several enzymes in small amounts. No single one of them is present in sufficiently large quantities to be considered a special feature of the cells.

ENZYMES IN POLYMORPHONUCLEAR LEUCOCYTES.

A comparison of the enzymes of the lymphocytes with those contained in the polymorphonuclear leucocytes was undertaken because many workers believe that the polymorphonuclear leucocytes may develop from the lymphocyte. The cells of the rabbit and cat were studied ; again for technical reasons the supply from the cat was much less than that from the rabbit.

Achalme (1899) first described a protease, a lipase and an amylase which he found in the cellular deposit of human pus. Jochmann and Müller (1906), using extracts of bone-marrow, found that the protease content of the polymorphonuclear leucocytes differed in the various species of animals. Those of the carnivorous animals contained trypsin, while those of the herbivora did not. The truth of this generalization has been questioned by Willstätter *et al.* (1930–32), who found trypsin in the leucocytes of the horse.

In the rabbit a weak protease acting in acid media has been found (Parker and Franke, 1917 ; Dernby, 1918 ; Weiss, 1927), a nucleoprotease (Dubos and Macleod, 1938) and also a lipase (Fiessenger, 1923).

The leucocytes of the horse have been shown by Willstätter *et al.* (1929–32) to contain cathepsin, trypsin, erepsin, amylase and maltase ; no lipase could be found by Mancini (1910).

The dog has trypsin (Willstätter *et al*, 1930 ; Opie, 1905 ; Jochmann and Müller, 1906), amylase and nuclease but no lipase (Tschernoruski, 1911) in its polymorphonuclear leucocytes.

In man, using the white cells from cases of myeloid leukaemia, Husfeldt

(1931) found cathepsin, trypsin and erepsin present. Morris and Boggs (1911), using leucocytes from the same source, said they contained a protease, an amylase and a lipase. Fiessenger (1923) described a nuclease in human polymorphonuclear leucocytes.

As in the case of the work on the enyzmes of the lymphocytes some of the discrepancies in the results of different workers may be explained by the impurity of their cell extracts. Another reason for the differences in the recorded results is the species differences in the cells pointed out by Jochmann and Müller (1906). It is difficult, therefore, to give a satisfactory account of the enzymes found in these cells. They have usually been distinguished from the lymphocytes by possessing trypsin and by the absence of lipase. This distinction, however, only holds good for certain animals.

Method of Collecting Polymorphonuclear Leucocytes.

A simple method for obtaining polymorphonuclear leucocytes, in large amounts and in a state of purity was first described by Haan (see Abderhalden, 1927). Warm normal sterile saline (200 or 300 c.c.) is run into the peritoneal cavity of an adult rabbit ; four hours later the fluid is withdrawn with a cannula, and is found to be opalescent owing to the presence of large numbers of polymorphonuclear leucocytes. The fluid was collected in 5 c.c. of 4 per cent. sodium citrate in order to prevent clotting. A smear was always made of the fluid and stained both with neutral red and Leishman's stain, and a differential cell count carried out. The results of these counts invariably showed the polymorphonuclear leucocytes to be present in amounts from 95 to 98 per cent.

With the cats the method did not work so successfully. Very strict aseptic precautions had to be observed. There was no difficulty in running in the saline, but there was considerable difficulty in withdrawing reasonable amounts. It appeared that the thick omentum adhered to the original puncture wound and blocked the cannula when it was inserted for the second time. The cat always appeared to be more upset than the rabbit by the procedure ; even though the saline was given warm the animal invariably had a severe rigor coming on soon after the injection and lasting about 15 mins.

A more serious drawback to the use of cats was that the character of the cells altered ; whereas in the rabbit after eight successive injections the cells still remained over 90 per cent. polymorphonuclear leucocytes, in the cat by the third injection the value had sunk to 50 per cent. The remainder of the cells appeared to be lymphocytes. It was also found that a number of red blood cells was always present when the saline was withdrawn, and this was also observed when aleuronat was given to cats for the same purpose (Kling, 1910).

Results.

The methods used for testing the enzymes in the polymorphonuclear leucocytes were the same as those described for the lymphocytes. In the cells of the rabbit the following enzymes were found :

(*a*) A cathepsin acting at pH 3·5. No trypsin was detected.

(*b*) A nuclease which split 30 per cent. of a 1 per cent. solution of thymonucleic acid in 24 hrs. at 37° C. at pH 4·6, but not at pH 9·0.

(*c*) A lipase capable of splitting completely a saturated watery solution of tributyrin in 4 hrs.

It thus appears that the polymorphonuclear leucocytes of the rabbit contain enzymes with actions identical with those of the lymphocytes ; they appear, however, to be present in larger amounts.

In the cells of the cat the following enzymes were found, but in this case there was much less material available and the results were not so complete.

(*a*) A trypsin acting at pH 8·0 is present in relatively large amounts ; there is also a weak cathepsin acting at pH 3·5.

(*b*) No nuclease is present.

(*c*) No lipase is present.

There is, therefore, a difference in the enyzme content of the lymphocytes and the polymorphonuclear leucocytes of the cat ; and again between the polymorphonuclear leucocytes of the rabbit and the cat.

Special enzymes.

In addition to the investigation into the presence of the more commonly occurring enzymes in the leucocytes, it was decided to test their action against a few physiologically active substances known to be destroyed by special enzymes in certain parts of the body.

The substances chosen were adenosine, acetyl-choline and histamine. Most of the work was done on the first of these.

Adenosine.—This substance was first described by Drury and Szent-Gyorgyi (1929), and Bennett and Drury (1931) stated that they had evidence that it was destroyed in the liver and lungs of the cat. Later Drury *et al.* (1938) showed that in the rabbit it was the red cells of the blood that destroyed the adenosine, while in the cat it was the plasma. More recently Conway and Cooke (1939), studying the deamination of adenosine by extracts of fresh rabbit tissues, found that the maximum action occurred with extracts of the appendix, the spleen and the Peyer's patches. All these three tissues contain large numbers of lymphocytes. The method used was similar to that described by Bennett and Drury (1931), whereby the adenosine is estimated by measuring the duration of the heart-block produced on injecting the substance into the guinea-pig's heart.*

The guinea-pig preparation was first standardized until a given dose of adenosine, usually about 20γ, produced a heart block of constant duration. At first, as the animal cooled, it became rather more sensitive to the adenosine, but afterwards a good preparation would last for about two hours. Injections were given every few minutes, sufficient time being allowed between them to enable complete aeration of the blood to take place. The standard dose of adenosine was always given after every test injection to ensure that the preparation was reacting satisfactorily. An approximately 1 per cent. extract of the cells or tissues to be tested was made up in saline, and kept in a water bath at 37° C.. A quantity of adenosine was then added to make up a concentration

* I am indebted to Dr. B. G. Maegraith for showing me the guinea-pig heart preparation.

of 200γ per c.c., and at suitable intervals 0·1 c.c. was removed and injected into the cannula.

A large number of tissue extracts were tried, and it seemed to make no difference whether the tissues were used fresh, or after rapid drying in an evacuated desiccator. Table I gives a summary of the results obtained with the tissues of the rabbit. It can be seen that both the lymphocytes and the polymorphonuclear leucocytes, and also those tissues that contained them, were able to destroy adenosine more rapidly than other tissues.

TABLE I.—*Adenosine-splitting Activity of Rabbit Tissues.*

Tissue extract used (rabbit), 1 c.c. of 1 per cent. extract + 200γ adenosine.	Time to destroy adenosine at 37° C. (min.).
Lymphocytes (pure)	3–5
Polymorphonuclear leucocytes (pure)	,,
Lymph glands	,,
Spleen	,,
Bone marrow	,,
Thymus	10
Pancreas	15
Liver	,,
Kidney	,,
Lung	,,
Skeletal muscle	>30
Red blood corpuscles	>60 (not destroyed)

The experiments were then repeated using extracts of the same tissues from the cat. In this animal, however, all the tissues, with the exception of skeletal muscle, destroyed adenosine with great rapidity, and no differences could be detected by this method. The spleen appeared to be most powerful, and 1 c.c. of 1 per cent. extract could destroy 600γ of adenosine almost instantaneously.

Acetyl-choline.—This drug has the same effect on the guinea-pig's heart as adenosine (Bennett and Drury, 1931), and in this case the same preparation was used. Unfortunately the animal dies more quickly because the bronchial spasm induced by the drug prevents adequate aeration of the blood. The dose required is much smaller and 1γ will usually produce a satisfactory heart-block.

In the rabbit neither lymphocytes nor polymorphonuclear leucocytes used as 1 per cent. extracts had any effect on 10γ of acetyl-choline in 1 hr. at 37° C. Red blood cells destroyed the same amount in about 45 mins.

Histamine.—The drug was detected by its effect on the isolated guinea-pig's intestine, but again neither the lymphocytes nor the polymorphonuclear leucocytes had any destructive action on the solutions of histamine.

It appears from these results that in the rabbit the lymphocytes share with the polymorphonuclear leucocytes the power of destroying adenosine in the body. I was unable to confirm the findings of Drury *et al.* (1938) that the red

cells of this animal are able to destroy it. The power of the lymphocytes to destroy adenosine explains the results of Conway and Cooke (1939).

This is also another good example of the similarity of the enzymes of the polymorphonuclear leucocytes and lymphocytes of the rabbit.

In the cat it was not possible to show that the lymphocytes played a particularly important part in the destruction of adenosine, for in this animal a large number of tissues are able to destroy it very rapidly. The lymphocytes of the rabbit do not have any action on acetyl-choline, but in the cat it was found that extracts of lymph glands were able to destroy acetyl-choline more rapidly than other tissues.

The Action of the Leucocytes against Bacteria and the Relationship between Lysozyme and the Leukins.

Another special property of the white blood corpuscles, particularly the polymorphonuclear leucocytes, is their power to destroy bacteria. Dubos (1937) and Dubos and Macleod (1938) showed that the enzyme responsible for the autolysis of pneumococci was a nucleo-proteinase. They could obtain this enzyme from the polymorphonuclear leucocytes of the rabbit as well as from other tissues. In the early part of the century, however, there appeared a large number of papers describing the bactericidal action of the polymorphonuclear leucocyte extracts on certain organisms. The name "leukin" was given to the agent believed to be responsible for this action (Pettersson, 1905).

The method used to detect the killing of the bacteria was that of colony counting, and there is little mention of the common errors usually associated with the use of this method. The results on the whole were very inconsistent. Table II summarizes those obtained by different workers.

It can be seen that the bactericidal action of the leukins was noted consistently against *B. subtilis*, *B. anthracis* and the staphylococci.

Fleming (1922, 1929, 1932) and others have tested the action of lysozyme against different bacteria, but as in the case of the work on the leukins the results reported have been inconsistent (Melnik, 1936). *B. subtilis*, *B. anthracis* and the Sarcinae are regularly killed by lysozyme, the last group being lysed as well, whilst against streptococci, staphylococci, pneumococci, *Proteus* and *Bact. typhosum*, some have reported killing and others have found it to have no action (Melnik, 1936).

In discussing the nature of the leukins, attention has been drawn to the similarity between their properties and those of lysozyme (Ledingham, 1929). Levaditi (1914) gave an account of the properties of the leukins, and stated that they were stable in solution, could withstand drying, and heating up to 70° C. He added that they behaved like proteins, were precipitated by alcohol and inactivated by certain colloids; and finally that they acted better in acid than in alkaline media. These properties are all possessed by lysozyme (Fleming, 1932; Yermoliev and Bouianowskaia, 1938; Roberts, 1938).

Knorr (1929) also pointed out that the leukins act best against Gram-positive organisms; this is another feature of lysozyme (Fleming, 1932).

Mr. L. A. Epstein carried out some quantitative estimations of the amount of lysozyme in the various leucocytes, the results being summarized in Table III.

TABLE II.—*Summary of the Action of the Leukins on Different Bacteria as Obtained by Various Authors.*

Organism.	Sensitivity to leukin.	Animal supplying the leucocytes.	Author.
B. subtilis	+	Rabbit	Kling, 1910.
,,	+	Guinea-pig	Weil, 1911.
,,	+	,,	Pettersson, 1905.
B. anthracis	+	Rabbit	Kling, 1910.
,,	+	,,	Pettersson, 1908.
,,	+	Guinea-pig	Tsuda, 1909.
Bact. typhosus	—	,,	Pettersson, 1905.
,,	—	,,	Schneider, 1909.
,,	—	Rabbit	Werbitzki, 1909.
,,	+	,,	Mainwaring, 1912.
,,	+	Horse	,, 1912.
,,	(∓)	Rabbit	Zinsser, 1910.
,,	+	,,	Schneider, 1909.
Bact. paratyphosum A	+	,,	Werbitzki, 1909.
,, *B*	—	,,	,, 1909.
V. cholerae	±	Rabbit and guinea-pig	Pettersson, 1905.
,,	+	Guinea-pig	Weil and Toyosumi, 1909.
,,	+	,,	Nanukawa, 1909.
Staphylococcus (pathogenic)	+	Rabbit	Zinsser, 1910.
Staphylococcus	+	,,	Werbitzki, 1909.
,, (air borne)	+	Rabbit and guinea-pig	Kling, 1910.
Ditto	+	Guinea-pig	Weil, 1911.
Streptococcus	+	,,	Toyosumi, 1909.
,,	+	Rabbit	Pettersson, 1908.
,,	—	,,	Werbitzki, 1909.
Pneumococcus	+	,,	Pettersson, 1908.
Proteus	+	,,	,, 1908.

TABLE III.—*Lysozyme Content of Various Leucocytes and Lymphocytes.*

	Dilution of a 1 per cent. solution of dried leucocytes in which a complete lysis of *M. lyzodeikticus* is observed.
Rabbit lymphocytes	1 : 16
,, polymorphonuclear leucocytes	1 : 1600
Cat lymphocytes	1 : 16
,, polymorphonuclear leucocytes	1 : 16

The striking feature is the high figure obtained for the polymorphonuclear leucocytes in the rabbit.

A comparison was then made of leukin and lysozyme against a series of organisms under the same conditions, using a method that was free from the objections of the colony-counting method. This method consisted of observing the effects of the addition of these bactericidal substances to suspensions of bacteria in a Warburg respirometer.

About 1 c.c. of an 18-hr. culture of the organism to be tested was placed in the Warburg vessel with strong soda in the central chamber, and into the side arm was placed 0·5 c.c. either of a 0·1 per cent. solution of pure egg lysozyme prepared according to Roberts' (1938) method, or a 1 per cent. extract of the leucocytes. This extract was made by adding 10 mg. of the dried powdered cells to 1 c.c. of distilled water and leaving it for an hour at 37° C. It was found to be unnecessary to freeze and thaw the mixture in order to extract the bactericidal agent from the cells.

The oxygen uptake of the bacteria was then observed, and when it had become steady the contents of the side arm were added to the central part of the vessel. On sensitive organisms the effect was to bring about an abrupt and complete cessation of the oxygen uptake of the bacteria. In all experiments a control vessel was set up containing water in the side arm which was mixed at the same time as the other vessel, and another control vessel was used and left alone in order to see that the respiration of the bacteria was maintained.

The leucocytes used were almost entirely those from the rabbit, but against two organisms found to be sensitive to their action, a similar extract of the polymorphonuclear leucocytes of the cat was tried. Results are summarized in Table IV.

TABLE IV.—*Action of Leucocyte Extract and Lysozyme on Respiration of Bacteria.*

Organism.	Lysozyme (0·1 per cent. solution).	Extract of rabbit leucocytes (1 per cent.).	Extract of cat leucocytes.
B. subtilis	+	+	—
M. lysodeikticus	+	+	—
Sarcina	+	+	..
Staphylococcus (from skin)	±	±	..
Staph. aureus (pathogenic)	—	—	..
Staph. albus (pathogenic)	—	—	..
Bact. lactis aerogenes	—	—	..
Proteus (3 strains)	—	—	..
Ps. pyocyanea	—	—	..
Bact. coli	—	—	..
Bact. typhosum	—	—	..
Bact. paratyphosum B	—	—	..
Bact. shigae	—	—	..
Bact. flexneri (3 strains)	—	—	..
Bact. aertryke	—	—	..
Bact. enteritidis (two strains)	—	—	..

+ indicates that the O_2 uptake ceased immediately on adding the test solution.
— indicates that the addition of the test solution had no effect on the O_2 uptake of the bacteria for at least 90 min.

Unfortunately the list is not as complete as is desirable, for it was found that all strains of streptococci and pneumococci tested did not take up any appreciable amount of oxygen even if blood was added as a source of catalase; their respiration, therefore, could not be measured by this method.

It will be seen that the results were uniformly negative for the action of lysozyme against all but the non-pathogenic bacteria. In the case of the pathogenic strains of the staphylococci six strains freshly isolated from human sources were tried with negative results. Similarly with *Proteus* and *Bact. flexneri* three separate strains were tested.

The action of lysozyme and the leukins ran parallel, and the cat's polymorphonuclear leucocytes which contain practically no lysozyme failed to kill even those organisms sensitive to the rabbit's leucocytes.

It has been found that the substrate upon which lysozyme acts is a polysaccharide, and that its action liberates acetyl-glucosamine and other hexose bodies which can be detected by their free reducing groups (Epstein, 1940). A purified fraction of this substrate was provided by Mr. Epstein, and incubated with extracts of polymorphonuclear leucocytes from the rabbit and the cat. The increase of the reducing power of the solution was measured after 24 hrs. at 37° C., and from this figure the percentage of the substrate split could be estimated. The cat's leucocytes split only 2 per cent., while the rabbit's cells split 15 per cent. of the substrate. The latter is equal to the amount of splitting obtained with a solution of pure lysozyme acting on this particular substrate.

It is reasonable to conclude from these data that the leukins are lysozyme.

SUMMARY.

Table V shows the enzymes that have been found in the leucocytes of the rabbit and the cat.

TABLE V.—*Summary of Enzymes Found in Leucocytes and Lymphocytes.*

Enzymes.	Rabbit.		Cat.	
	Lymphocytes.	Polymorphonuclear leucocytes.	Lymphocytes.	Polymorphonuclear leucocytes.
Cathepsin	+	+	+	+
Trypsin	—	—	—	+++
Erepsin	—	—	..	..
Nuclease	+	++	+	—
Amylase	+	++	..	..
Lipase	+	+	+	—
Lysozyme	+	+++	+	+
Adenosinase	++	++	..	..

The view held by some histologists that the polymorphonuclear leucocytes develop from the lymphocytes receives some support in the case of the rabbit from the fact that both types of cell contain the same enzymes. This reasoning is supported by no such evidence in the case of the cat.

The lymphocytes and the polymorphonuclear leucocytes of the rabbit contain an enzyme destroying adenosine, and it appears to be present in these cells in amount greater than in the other tissues of this animal.

The polymorphonuclear leucocytes of the rabbit contain a large amount of lysozyme. It has been shown that the bactericidal agent present in these cells hitherto called leukin is probably lysozyme.

This enzyme is present in small amounts only in the polymorphonuclear leucocytes of the cat.

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THE EFFECTS OF THE REMOVAL OF LYMPHOID TISSUE.

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A WELL recognized procedure for discovering the function of an organ is the study of the effects of its operative removal in animals. The difficulty of applying this method to the elucidation of the function of the lymphocyte is the fact that the lymphopoietic tissue is widely dispersed over the whole body.

According to Delamere (1903) extensive lymphatic ablations were performed by Ehrlich and Reinbach, who noted a drop in the blood lymphocytes afterwards. Unfortunately the reference was not given and it has so far proved impossible to trace the original work, if indeed it was ever published. Several

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writers have quoted Delamere but none give the original reference. It is quite likely that the following passage, quoted from Myers's translation of 'The Histology of the Blood' by Ehrlich and Lazarus (1900), may have inspired Delamere's statement:

"Ehrlich had previously observed that when extensive portions of the lymphatic glandular system are put out of action by new growths and similar causes, the number of lymphocytes may be considerably diminished. These observations have since that time been confirmed by several authors. For examples, Reinbach describes several cases of malignant tumour, particularly sarcoma, in which the percentage of lymphocytes, which normally amounts to about 25 per cent., was very considerably lowered; in one case of lymphosarcoma of the neck they only made up 0·6 per cent. of the total number. These conditions are quite easily and naturally explained by the exclusion of the lymphatic glands. . . ."

Two other references by Delamere (1903) to experimental ablations by Rokitski and by Tschigaieff are also unobtainable here as they were presented as theses at St. Petersburg some fifty years ago.

Less extensive ablations of the lymph glands have been done by many workers, but chiefly to see whether regeneration of the glands occurred or not —a matter on which there has been some controversy.

In the last 15 years of last century Bayer, Ribbert and others reported the regeneration of lymph glands from adipose tissue following operative removal in animals. Their results were criticized by Meyer (1906), who ascribed them to faulty technique and inadequate removal. Meyer himself was quite unable to find any new formation of lymph glands unless small pieces or minute glands had been left behind which could hypertrophy.

Vecchi (1911) was also unable to find any newly-formed lymph glands after complete extirpation in any area. Baum (1926) was likewise unable to find any new formation of glands, but described in detail the regeneration of lymphatic channels connecting the afferent and efferent vessels of the extirpated gland. Teneff (1934) agreed with these findings and confirmed them. He also found that autoplastic grafts of lymphoid tissue degenerated and did not grow into lymph glands. This observation, however, is at variance with that of Jaffé and Richter (1928), who obtained complete and functional regeneration of transplanted lymph glands.

Papilian and Russu (1934) reported the formation of large lymph glands along the greater curvature of the stomach in dogs after those originally present there had been removed. Their paper is not convincing and their technique, too, is rather suspect, as they regard splenectomy as a very dangerous operation. Petri (1925) thought that lymph glands could be generated if needed from fat tissue. Okubo (1936) also described newly formed glands in areas from which some had been removed. However, he left the capsules of the glands *in situ* and found regeneration of the parenchyma.

There seems to be fairly general agreement that small glands or fragments of glands left behind at operation can undergo hypertrophy. Those who state that new lymph glands cannot be formed from fat or other tissue ascribe the conflicting results of other people's work to faulty technique whereby some pieces of lymphoid tissue were inadvertently left behind. This is easily

possible on account of the lack of colour differentiation between the glands and the surrounding tissue which usually consists mostly of fat.

Rouvière and Valette (1937) reviewed the literature on the subject and themselves performed experiments on the popliteal glands in 50 rabbits. They used methylene blue injected into the pads of the feet to show up the lymphatics and the glands. In no case did they find more than one gland in the fossa. In none of their cases in which complete extirpation of the gland was done did they find any regeneration. Forty of their cases were examined one to three months after operation.

EXPERIMENTAL.

In the experiments to be described, successful attempts were made to remove very large amounts of the total organized lymphoid tissue. The blood picture was followed in many of the animals and all were subjected to histological examination post-mortem. Some rabbits were used because of the large amount of information already available about their lymphoid tissue (Hellman, 1914) and their blood (Sjövall, 1936; Pearce and Casey, 1930), but most of the experiments were on rats, as they stand extensive operations very well, although subject to intercurrent respiratory disease.

The technique used was that found satisfactory by Florey and Pullinger in preliminary experiments. Pontamine sky blue (Gurr's 6BX), a relatively non-toxic vital dye, was injected seven to ten days before operation. In the interval the dye was absorbed and then largely removed from the circulation by the cells of the reticulo-endothelial system. As these are abundant in the lymphoid tissue the lymph glands soon became blue and after a few days, when the dye had disappeared from the other tissues, could be very easily identified. Even minute nodes of pinhead size could be seen without difficulty, though they would certainly have been missed without the dye. The dye was given as a 5 per cent. solution in distilled water, autoclaved after preparation; rats were given 1 c.c. per 100 g. body-weight subcutaneously and rabbits 5 c.c. per kg. intravenously. No ill-effects were observed from the dye in these dosages, though one rat died after treble the usual dose. No alteration of the blood picture was seen as a result of the pontamine.

Note on Bartonella infection in rats.

Rats in which this infection is present develop a profound anaemia when subjected to splenectomy (Lauda, 1930). About 70 per cent. recover and are then immune to further infection. Perla and Marmorston (1936) showed that this immunity was accompanied by the appearance of nodules of lymphoid tissue in the liver. As splenectomy was one of the operations necessary for a complete lymphadenectomy, either a Bartonella free strain had to be used or the infection had to be cured. The latter choice was adopted and the method was that used by Mayer, Borchardt and Kikuth (1927). When the rats were received from the stock room they were given 2 mg. neo-salvarsan subcutaneously per 100 g. body-weight and were subsequently kept apart from infected rats. No rats receiving this treatment developed anaemia after splenectomy.

Operative Technique.

Rats.

Four- to six-months-old rats treated with salvarsan and pontamine had three blood counts and were then subjected to a series of operations in which as much of the organized lymphoid tissue as possible was removed. The areas operated on were: Abdominal cavity (including splenectomy), neck, axillae, popliteal fossae, inguinal regions, thorax (including thymectomy).

The positions of the glands varied a little from those described by Job (1915) in the Norwegian rat and by Donaldson (1924). Fig. 1 is a composite diagram showing the positions of the glands found in our albino rats.

Two operations only need special description, as none of the others present any real difficulty, though many need considerable care and practice (e.g. the removal of the glands behind the portal vein). Ether anaesthesia was used for all operations.

Thymectomy and removal of thoracic glands.—Positive pressure anaesthesia was used after inserting an endotracheal tube under direct vision. This tube was made from a lumbar puncture needle No. 16 S.W.G. A pressure of + 2 cm. mercury was found sufficient for inflation of the lungs. A rubber side tube close to the needle was open to the air and could be occluded at will by the anaesthetist to raise the pressure.

The sternum was exposed by a midline incision and the muscles dissected off it, using the electrocautery to prevent haemorrhage. The sternum was cut across in the second interspace after putting two "ligatures" of linen thread above and below. These were used for retraction and for pulling the cut ends of the bone together afterwards. The intercostal muscles of the second space were cut outwards as far as necessary. By using the second space there was no trouble from the internal mammary artery which, when the third space was used, had to be cut between ligatures. In the third space access to the deep thoracic glands was rather easier but thymectomy was more difficult. Although the breathing was sometimes noisy for a day or two after operation, the animals did not seem to be in any way incommoded by it and were able to run round their cages and to eat as soon as they were conscious.

Peyer's patches.—The convenient name of "peyerectomy" is suggested for the operation of removing these structures. This proved to be the most often lethal operation. Several different methods were tried. Although five rats were successfully brought through the several stages necessary, none of those that had already had all their other organized lymphoid tissue removed survived. After a number of casualties it was regretfully decided not to try any more peyerectomies in lymphadenectomized animals.

The rat has about 20 patches, of which all but three or four are in the small intestine. The highest one is usually at, or just below, the duodeno-jejunal junction in the least mobile part of the gut. From this point to the end of the small intestine they occur at more or less evenly-spaced intervals of about 2 to 4 cm. Each consists of some 4 to 20 follicles that can easily be seen with the naked eye even in unstained animals. For demonstrating them as a permanent preparation a modification of Hellman's (1922) method was worked out. In the caecum there are always one or two patches near the tip.

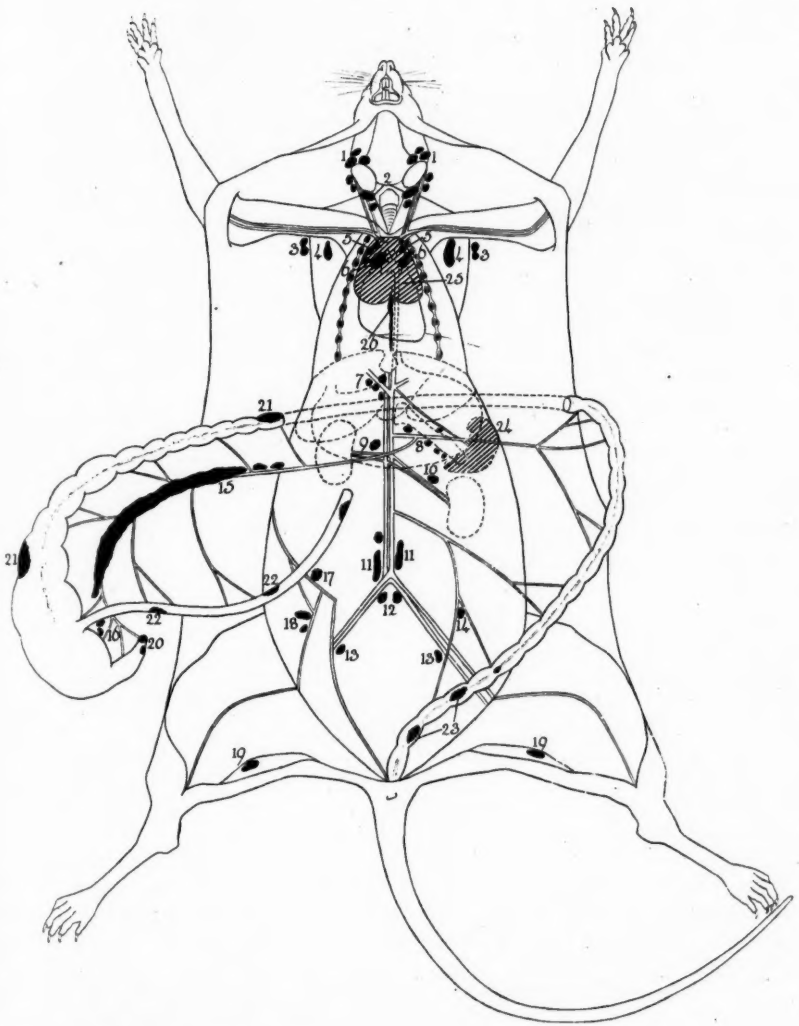

FIG. 1.—The lymph glands of the rat.

1. Superficial cervical.
2. Deep cervical.
3. Posterior axillary.
4. Internal axillary.
5. Superficial mediastinal.
6. Deep mediastinal.
7. Portal.
8. Postgastric.
9. Right renal.
10. Left renal.
11. Para-aortic.
12. Sacral.
13. Iliac.
14. Mesocolic.
15. Mesenteric.
16. Caecal.
17. Posterior abdominal wall.
18. Inguinal.
19. Popliteal.
20. Peyer's patch in caecum.
21. Ditto in large intestine.
22. „ in small intestine.
23. „ in rectum.
24. Spleen.
25. Thymus.
26. Mediastinal organ.

There is another constant one a few centimetres along the colon, usually one halfway along and always one near the rectum.

(1) The patch was seized with Graafe forceps and a silk or fine catgut ligature tied round the "pedicle" formed by lifting it up. The patch was then excised and the stump containing exposed mucosa either touched with pure carbolic or cauterized with the electrocautery. The latter was found the more successful method. It was also found useful to tie an omental graft, preferably not detached, over the wound.

(2) The next method was devised to overcome the difficulty of dealing with the exposed bacteria-laden mucosa. The patch was tied off from inside so as to leave the two serosal surfaces in contact. A series of patches, usually five, were selected and the central one incised. Through the hole a specially made pair of very fine forceps with toothed ends was passed. The highest of the selected patches was grasped from within and gently pulled down, causing an intussusception, till it was outside the hole. The extreme laxity of the rat's mesentery made this manoeuvre possible. A silk ligature was tied round the base of the patch, which was then excised and the intussusception reduced by gentle manipulation. The remaining patches were dealt with in the same way except for the incised one, which had to be removed by the first method. Usually the small intestine was done in two stages at a fortnight's interval and the large intestine done another two weeks later.

Rabbits.

The broad plan of the series was similar to that used for the rats, but anatomical differences imposed certain modifications, two entirely different operations, appendicectomy and "sacculectomy," having to be added to the list. Fig. 2 is a composite diagram showing the position of the rabbit's lymph glands and related organs.

Sacculectomy.—This is the name proposed for the operative removal of the sacculus rotundus, which is a collection of lymphoid tissue in the caecal wall at the point of entrance of the ileum. In appearance and structure it is similar to the appendix. Its removal presents difficulties.

The sacculus is a pouch some 3 to 4 cm. in diameter. Two deep sulci extend round it for two-thirds of its circumference and contain blood vessels which supply not only the sacculus but also the adjacent caecal wall. It was the enforced interference with these vessels that caused the operative difficulties and several of the fatalities.

The ileum was cut between ligatures about 1 cm. from its termination. The upper end was invaginated through a purse-string suture. The caecum was transected through the segment next to the sacculus on the appendicular side. The colon, which here lies alongside with a common mesentery and blood-supply, was also transected at the same level. Both these manoeuvres were done "aseptically" between clamps. The ends were then insewn by the Parker-Kerr technique so that no soiling of the peritoneum occurred. Side-to-side anastomoses were done between the colon and caecum and the ileum and caecum. Great care was taken to conserve the blood supply to the parts not removed.

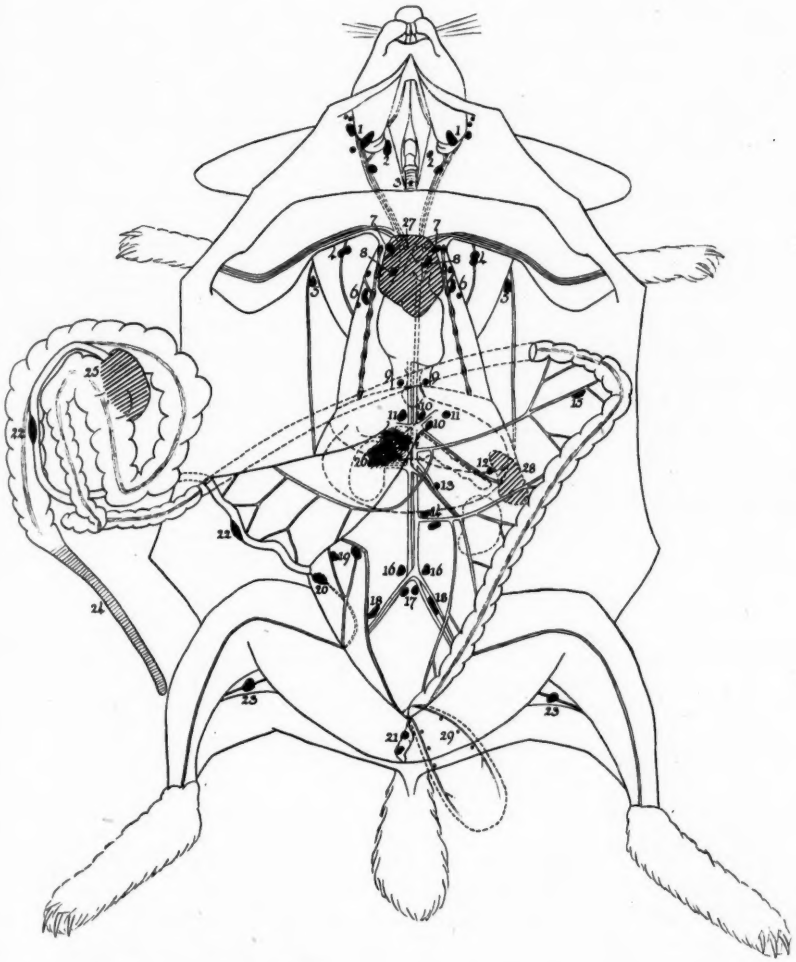

FIG. 2.—The lymph glands of the rabbit.

1. Superficial cervical.
2. Deep cervical.
3. Pretracheal.
4. Mid-axillary.
5. Posterior axillary.
6. Anterior axillary.
7. Anterior thoracic.
8. Posterior thoracic.
9. Para-xiphoid.
10. Portal.
11. Anterior gastric.
12. Pancreatic.
13. Renal.
14. Mesenteric.
15. ,,
16. Para-aortic.
17. Pelvic.
18. ,,
19. Anterior inguinal.
20. Posterior inguinal.
21. Caudal.
22. Peyer's patch.
23. Popliteal.
24. Appendix.
25. Sacculus rotundus.
26. Pancreas Aselli.
27. Thymus.
28. Spleen.
29. Posterior vesical.

The Effect of Extensive Lymphadenectomy on the Blood Count.

The blood counts were followed before, during and after the series of operations in seven rats. At the beginning of the experiments these rats were from 92 to 111 days old and the survival periods after the last operation varied from 58 to 268 days. In all instances the rats remained in good health throughout the time and all but one gained weight normally.

Each rat had at least three pre-operation counts and their blood lymphocytes varied from 9600 to 13,300, average 11,330 per c.mm. Post-operatively their counts varied from 3600 to 13,300, average 8470. The pre- and post-operation differences varied from a fall of 9700 to a rise of 1800 ; the average was a fall of 3060.

Two thymectomy controls showed no significant alteration in the blood lymphocyte level and five peyerectomy controls had differences ranging between a rise of 11,500 and a fall of 5400, with an average rise of 3000 cells per c.mm.

The splenectomy controls, of which there were six, all showed a fall of 800 to 6700 with an average of 4100, which is nearly 1000 more than that shown by the extensively lymphadenectomized animals.

In the rabbit the chief source of supply of lymphocytes is the mesenteric mass of glands known as the pancreas Aselli. Thus in 5 rabbits (average initial blood lymphocytes 6800 per c.mm.) the lymphocyte count fell by amounts from 2862 to 417 per c.mm. (average 2000) after the operation ; in four out of the five cases the fall is significant. Splenectomy performed later did not further alter the blood lymphocytes by a significant amount.

In the rabbits there was often quite a marked and prolonged fall after sacculectomy but as this usually interfered with the animals' nutrition the fall may have been due to this factor and not to the removal of the sacculus as such. Thus it appeared that both in rats and rabbits it was not possible to lower very considerably the circulating lymphocytes by ablation of large amounts of lymphoid tissue.

In both rats and rabbits it was noticed that, should sepsis occur, the blood lymphocytes also participated in the leucocytosis, though this mainly affected the polymorphs.

Results of "Peyerectomy."

The actual numbers of Peyer's patches removed operatively and found at post-mortem are given in Table I.

TABLE I.—*Results of "Peyerectomy."*

	Patches removed operatively.		Patches found at P.M.	
Rat No.	Small gut.	Large gut.	Small gut.	Large gut.
50	14	2	1	2
77	20	3	0	2
86	16	3	0	0
90	20	3	0	1
91	19	2	1	1

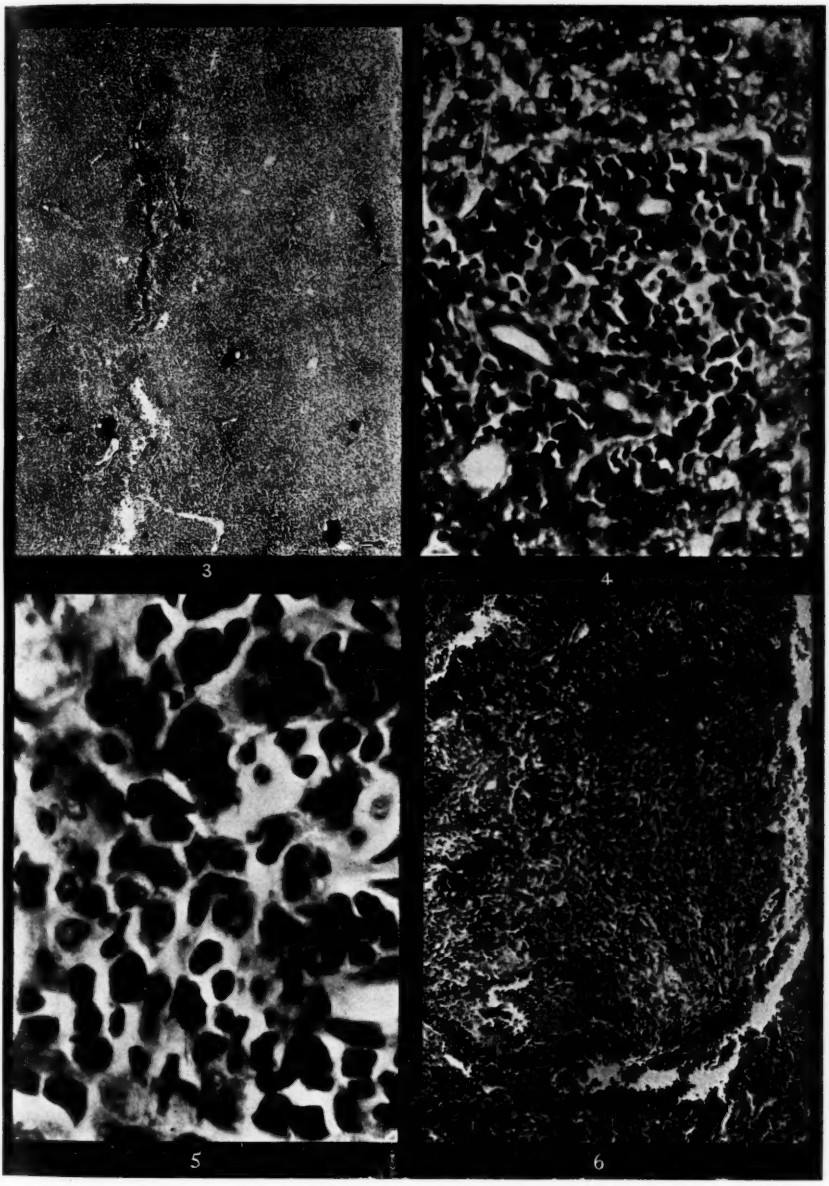

FIG. 3.—*Rat.* Low-power view of liver showing accumulation of lymphocytic tissue in portal tracts following lymphadenectomy, etc.

FIG. 4.—*Rat.* Liver after lymphadenectomy ; lymphoid nodule. × 420.

FIG. 5.—*Rat.* Part of same nodule as Fig. 4, × 1050. Shows mitoses of cells.

FIG. 6.—*Rat.* Lymph gland found P.M. Reticulum cell hyperplasia after extensive lymphadenectomy. × 114.

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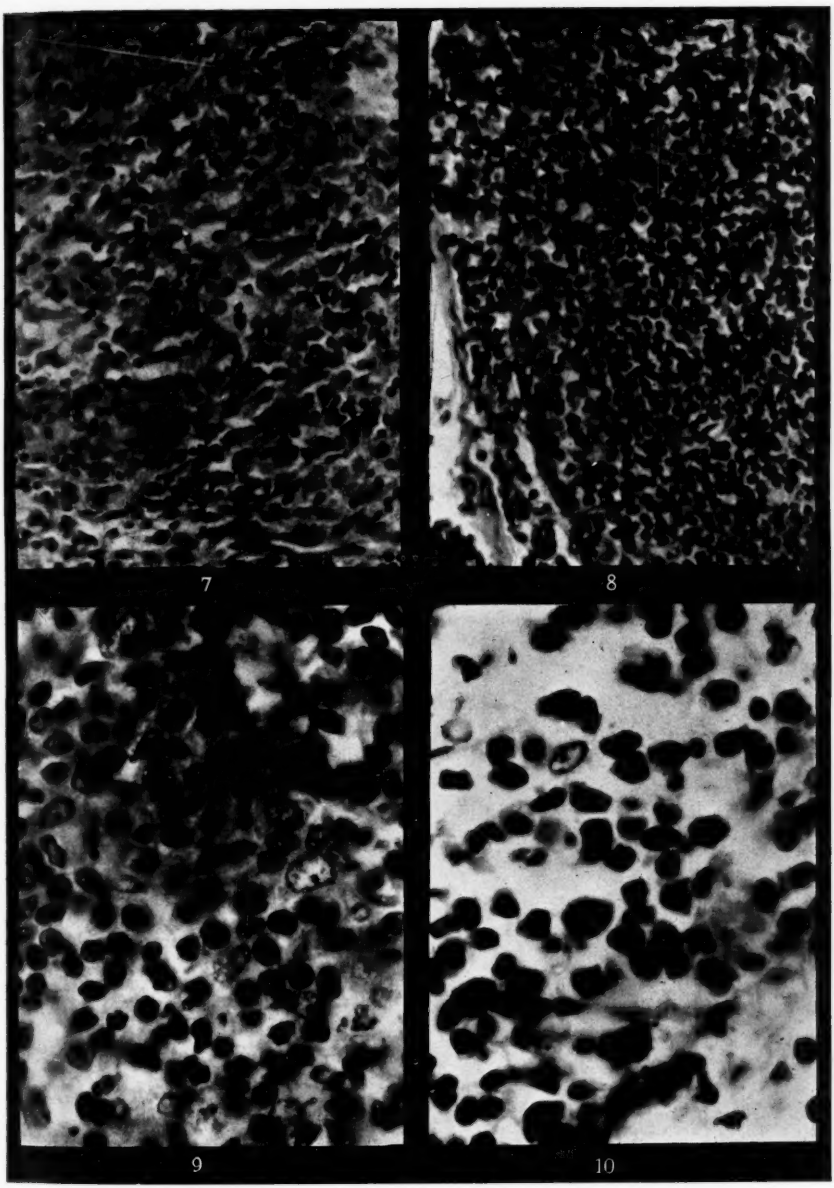

FIG. 7.—*Rat.* Same gland as shown in Fig. 6. Reticulum cell hyperplasia. × 420.

FIG. 8.—*Rat.* Lymph gland found P.M. Numerous lymphoblasts in cortex. × 420.

FIG. 9.—*Rat.* The same as Fig. 8. × 1050.

FIG. 10.—*Rat.* Lymph gland found P.M. in lymphadenectomized animal. Shows plasma cells. × 1050.

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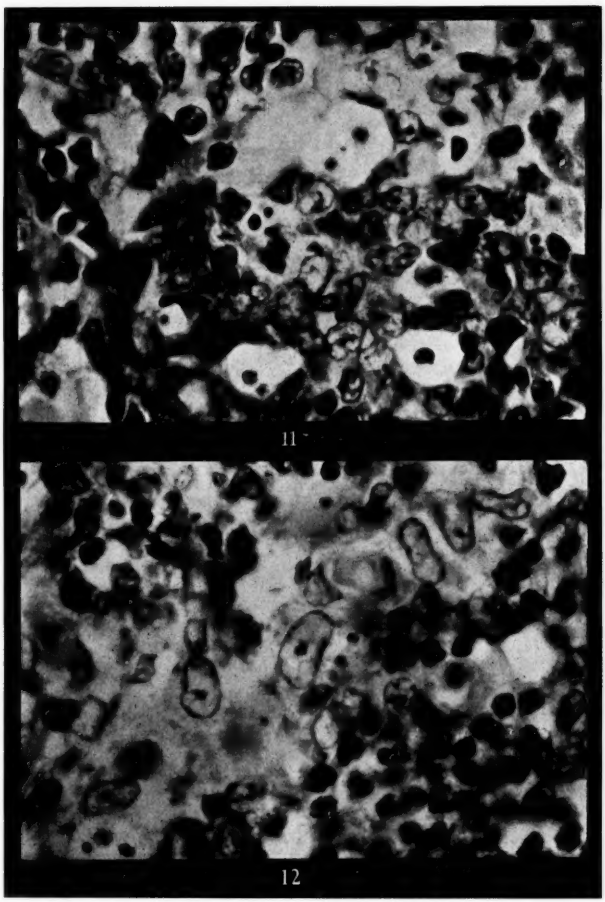

FIG. 11.—*Rabbit.* Lymph gland found P.M. after lymphadenectomy. Very active cell division. Large lymphocytes and reticulum cells. × 1050.

FIG. 12.—*Rabbit.* Lymph gland found P.M. after lymphadenectomy. Primitive and undifferentiated mesenchyme cells. × 1050.

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The small number found post-mortem seem to indicate that there is no regeneration or new formation of patches; those that were found had in all probability been overlooked at operation.

Regeneration.

The lymph glands did not regenerate after complete extirpation. Occasionally a few small glands were found post-mortem but perusal of the notes referring to the corresponding operation often showed that the number or weight of glands removed had been less than normal. Sometimes a note had been made, such as "Unsuccessful search made for . . . gland" at the time of operation and the post-mortem revealed a gland in or near the expected place. This absence of regeneration is in accord with the findings of most other recent workers and has already been discussed.

Histology.

Susa fixative was used for most rat and rabbit tissues, though Zenker-formol for the intestine and Bouin for the bone marrow were found to be rather better. All sections were stained with haematoxylin and eosin as a routine. For showing nuclear detail Heidenhain's iron haematoxylin was used. For showing the reticular structure of lymph nodes Robb-Smith's (1938) silver impregnation method was found satisfactory.

Rats.

The rats examined histologically comprise:

(1) The eight "complete" lymphadenectomies on which counts were done. In four of these the lymph glands were weighed as they were removed and the total compared with those found post-mortem. Even without allowing for any growth or hypertrophy of those left behind it was calculated that from 87 to 93 per cent. of the lymphoid tissue as well as that in the spleen had been removed. The thymus was also ablated, but not the Peyer's patches. In some of the later experiments even better clearances were obtained.

(2) Six rats that had been partly lymphadenectomized and had died at the last operation (usually thymectomy).

(3) Five completely lymphadenectomized rats, two of which had had three doses of T.A.B. vaccine and were killed one and two weeks later, and three which received normal horse serum in doses of 0·1, 0·2 and 0·3 c.c. intravenously ten days before death.

(4) Two thymectomy controls.

(5) Five "Peyerectomy" controls.

(6) Seven splenectomy controls.

(7) Six normal rats of various ages.

(8) Two rats, completely lymphadenectomized, in which about two-thirds of the liver was removed ten days before death by the method of Rous and McMaster (1924).

(9) Control normal rats for the hepatectomy, serum and T.A.B. injections.

The liver (see Figs. 3, 4 and 5).—Histological changes were found in the

liver ; they were similar to those described by Perla and Marmorston (1936) after splenectomy in rats infected with *Bartonella muris*. Masses of newly formed lymphoid tissue were found along the branches of the portal system. The type and arrangement of the cells suggested the medullary cords of the lymph glands. In none of the livers in which these masses of lymphoid tissue occurred were any " Flemming's nodules " found. Mitoses of the large pale cells (reticulum cells) were sometimes seen. The cells present were of the same types as those seen in lymph glands. Small lymphocytes predominated and were scattered among reticulum cells.

No such large masses of lymphoid tissue were found in any of the normal animals nor in the thymectomy nor " Peyerectomy " controls. Especially important is the fact that they were not seen in any of the seven splenectomized control rats. In a few of the control animals occasional *small* collections of lymphocytes were found in the same situations. There was no difficulty, however, in recognizing the characteristic excess in the lymphadenectomized animals. In the partially lymphadenectomized rats some showed large masses in the liver with mitoses in them, while others showed only small collections of cells.

The injection of horse serum and T.A.B. vaccine were given to see whether the characteristic mononuclear cells, which appear after the administration of foreign protein and which Rich (1935) believed to be lymphocytes, appeared in the nodules of new lymphoid tissue in the liver. There was, however, no marked difference in the nodules in the animals so treated when compared with other lymphadenectomized animals.

The hepatectomies were done to see whether in the regenerated liver, which takes about ten days to grow to the size of the portion removed, there would be any new lymphoid tissue. None was found in these two animals, but a larger series would be needed to establish the fact that lymphoid tissue was of slower growth than liver tissue.

The lungs of both normal and control animals contained small amounts of peribronchial lymphoid tissue in patches, especially at the hilum. In the lymphadenectomized rats this lymphoid tissue appeared to be hypertrophied and often contained typical " Fleming's nodules," which were, however, also present in some of the control animals. Without resorting to an elaborate quantitative technique, such as that used by Hellman's pupils at Lund, one can only record the opinion that there appeared to be quite a marked hypertrophy of the lymphoid tissue associated with the bronchial tree in lymphadenectomized animals.

The bone marrow did not show any marked constant change in the experimental animals. In some there was an apparent increase in the number of cells indistinguishable from small lymphocytes (haemocytoblasts of the unitarians). These were usually associated with larger pale cells similar to the reticulum cells of the lymph glands and other cells like medium lymphocytes. Small areas of marrow were found nearly devoid of maturing granulocytes and red cells, and these could have been mistaken for parts of a lymph gland. The number of eosinophils and the corresponding myelocytes, always a conspicuous feature of the rat's marrow, seemed also to be increased in the lymphadenectomized animals.

One can therefore say that in some rats there appeared to be lymphocytopoiesis going on in the marrow but that this was neither a constant nor a conspicuous feature of the sections.

The intestine did not show any increase, either to the naked eye or microscopically, of its associated lymphoid tissue. Unfortunately no lymphadenectomized animals were successfully Peyerectomized as well, in spite of repeated attempts. The nodules forming the patch resembled the Flemming's nodules of the lymph glands and often contained cells in mitosis. These findings were common to the lymphadenectomized animals and to the controls and there did not appear to be any appreciable difference between them.

Other tissues.—The following organs were examined in a number of animals in all series without finding anything abnormal: Skin, subcutaneous fat, striped muscle, tongue, heart, brain, salivary gland, thyroid, pituitary, sympathetic ganglia, suprarenal, kidney, pancreas, gonads and mammary glands.

The lymph glands.—In no animals were all the lymph glands completely removed so that at post-mortem a few were always found. These showed marked signs of activity. The so-called germinal centres were sometimes conspicuous. Mitotic divisions of the primitive cells in them were quite numerous, mitoses were also common in other parts of the gland and were by no means confined to the germinal centres. In some glands there were areas of reticulum cell hyperplasia. One of the most striking changes was found in a number of glands; this consisted of a modification of the cortex, which appeared as a solid rind of primitive cells, presumably lymphoblasts—(Figs. 6, 7, 8, 9, and 10). Very primitive mesenchymal cells were also a prominent feature of these glands and were found in no particular situations. (Cells with large nuclei, poor in chromatin and with only a small amount of cytoplasm, were taken to be primitive cells.) Plasma cells were often numerous as well, particularly in one of the rats which had received T.A.B.

Rabbits.

The liver did not contain masses of lymphoid tissue around the portal tracts as it did in the rat, although there were usually a few scattered lymphocytes there.

The bone marrow in one rabbit only showed nodules of lymphoid tissue. These, however, were neither large nor numerous.

The lungs were examined in four cases; two of these appeared to have an excess of peribronchial lymphoid tissue, whereas the other two had no such excess. In the first two there was a good clearance of lymph glands but in the last two there was not.

The lymph glands which were found at post mortem were compared with those removed at the first operation. The normal glands showed the usual histological picture. There were plenty of small lymphocytes throughout the gland, not only in the cortical tissue and medullary cords, but also in the sinuses. Flemming's nodules were present in various stages of activity but were not particularly numerous.

In the lymphadenectomized animals the picture varied. In some the

Flemming's nodules were abundant and well marked. In most the sinuses were rather empty of small lymphocytes; these cells were also far less prominent in the cortex, which was composed of cells of the lymphoblast type similar to those found in the rat. In other instances there were areas of reticulum cell hyperplasia. Several glands from one rabbit showed congestion of the sinuses and blood vessels with red cells, the appearance being those of a haemolymph gland.

In a few animals there seemed to be an excess of eosinophils in the glands found post mortem compared with those removed earlier. This, however, was not a constant change found in all animals. The changes, in the main, were similar to those seen in rats and like them indicated excessive lymphocytopoietic activity (Figs. 11 and 12).

DISCUSSION AND SUMMARY.

(1) When lymph glands have been completely removed there is no local regeneration of them. Reports by others that such regeneration does occur are almost certainly due to faulty technique, whereby small glands or parts of glands are left behind at operation with subsequent hypertrophy. The technique of vital staining of the glands used in the present work considerably diminished the likelihood of such an occurrence. As previously noted, some other workers also concluded that the lymph glands could not be regenerated after complete removal. The criticism which could be directed against their work is that they only removed a few glands and did not provide any stimulus to the body to form new ones. Such a stimulus was provided in the present experiments by the extensive character of the lymphadenectomies. That this stimulus was adequate was shown by the fact that new nodules of lymphoid tissue were formed in each instance—in the liver in rats and in the lungs in rabbits.

(2) The "compensatory" new formations of lymphoid tissue in these situations show that such tissue is of great importance to the body. It did not appear in the splenectomy controls, though similar nodules appeared after splenectomy in Perla and Marmorston's (1936) *Bartonella* infected rats. Their rats developed *Bartonella* anaemia and recovered, whereas the present series of rats were cured of their latent *Bartonella* infection before splenectomy. From this it might be deduced that lymphoid tissue, and presumably the lymphocytes derived from it, are concerned with the protection of the body against infection. That lymphocytes may be able to neutralize certain bacterial toxins was recently suggested by Moor and Newport (1939), but their experiments need amplifying.

(3) The blood lymphocytes in the lymphadenectomy experiments usually diminished somewhat, but not always. In two rats the final level was actually a little above the original one, and even in a rat in which 93 per cent. of the organized lymphoid tissue as well as that present in the spleen but excluding Peyer's patches was removed, the blood lymphocytes only fell to a quarter of their previous value. The new nodules of lymphoid tissue then are evidently formed to maintain the supply of blood lymphocytes, though they may have other functions as well. The histological picture of the remaining lymphoid

tissue and of the newly-formed nodules in these animals is one of excessive lymphocytopoietic activity. From these experiments, therefore, additional evidence has been obtained that lymphocytes are "used" by the body in large numbers, though for what purpose still remains obscure.

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ON THE RELATIONSHIPS BETWEEN THE RESPIRATORY ACTIVITIES OF BACTERIA AND THEIR SENSITIVENESS TO SULPHANILAMIDE, *p*-HYDROXYLAMINO- AND *p*-NITRO-BENZENESULPHONAMIDE.

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In the course of reading on the subject of the sulphanilamides attention was drawn, through a paper published in America by Locke, Main and Mellon (1938), to the work of Mayer (1937). This work, although it is decidedly interesting and published in considerable detail, seems to have been largely overlooked in this country. Until very recently, e.g. in an extensive review (Whitby, 1938), in which nearly two hundred references are quoted, it is completely ignored. Mayer tested the whole series of oxidation products of *p*-aminobenzene-sulphonamide which were prepared for him by Oechslin (Mayer and Oechslin, 1937). These were: *p*-hydroxylaminobenzenesulphonamide, *p*-hydrazobenzenesulphonamide, *p*-azoxybenzenesulphonamide, *p*-nitrosobenzenesulphonamide and *p*-nitrobenzenesulphonamide. All were tested by determining their therapeutic effect when administered by mouth to mice infected with small doses of virulent streptococcus.

The observations related principally to the haemolytic streptococcus, although some were also made on the mould *Aspergillus niger*. The most significant of these observations were the following: the nitro-compound was definitely superior to all other oxidation products of *p*-aminobenzenesulphonamide and also to Prontosil. All were active in varying degree with the exception of hydrazobenzenesulphonamide, which was found inert.

When tested *in vitro*, however, by (*a*) determination of bacteriostasis in cultures of streptococci in 50 per cent. serum broth at 38° C., or (*b*) determination of the bactericidal effect in aqueous solutions to which inocula from a culture were made, it was found that the nitro-compound was practically devoid of activity, the amino- capable of showing some effect up to a dilution of 1 in 5000 and the hydroxylamino- 10 and 100 times more potent than the latter. From these observations the author concludes that the hydroxylamino-compound is the active one, and that the superiority of the nitro-compound *in vivo* is due to the fact that it is more readily reduced to the hydroxylamino- than the amino- is oxidized to this substance. In support of that contention he demonstrates that a solution of haemoglobin is much more rapidly transformed to methaemoglobin in the presence of the nitro- than in the presence of the amino-compound, and he considers it obvious that a preliminary formation of hydroxylamino-compound is essential to this conversion.

The only reason he had for not recommending the therapeutic use of the nitro-compound was its greater toxicity. This is stated as M.L.D. 5–10 mg. for a 20 g. mouse. Best therapeutic results in mice were obtained with a 2·5–5 mg. dose. The drug was administered 4–5 times *per diem*, but it is not quite clearly stated whether 5–10 mg. is the total or the 4-hourly tolerated dose.

Locke and his colleagues in America (Shinn, Main and Mellon, 1938, 1939 ; Main Shinn and Mellon, 1938 ; Locke, Main and Mellon, 1938, 1939 ; Locke, 1939) appear to have over-emphasized the inhibitory effect of hydroxylamino-compounds on catalase in explaining the action of the sulphanilamides. It may well be that accumulation of peroxide plays a part as is suggested by their observations, but it is doubtful whether inhibition of catalase is important, since the bacteriostatic action of these drugs can be demonstrated even when dealing with bacteria which do not produce catalase and are growing in media devoid of that substance : streptococcus, pneumococcus and various anaerobes.

So far as we are aware there has been no attempt to expand Mayer's work by observations on the bactericidal and bacteriostatic actions of the hydroxyl-amino- and nitro-compounds, although Bratton, White and Marshall (1939) have confirmed his observation of the superiority of the hydroxylamino-compound to sulphanilamide as a bactericidal agent for streptococcus *in vitro*. Also, as far as the results refer to sulphanilamide, they cannot be at all readily compared with results obtained in highly specialized conditions of culture as in Fleming's work (1938) and that of Osgood (1938). There are, however, a considerable number of observations on the *in vitro* potency of sulphanilamide when acting in relatively simple media, and without attempting an exhaustive survey of these, their trend is summarized by the following general statements :

1. Inhibitory and bactericidal effects have been demonstrated *in vitro* for streptococci, pneumococci, *Brucellae* and *Neisseriae* ; but staphylococcus, the coliform bacteria, *Corynebacteria* and anaerobes are not affected or are much less affected. (Some or all of these observations are made in the publications of the following : Colebrook, Buttle and O'Meara (1936), Buttle (1937), Bliss and Long (1938), Britton (1938), Felke (1938), Chinn (1938), Spray (1938) and Green (1940).

2. These bactericidal and inhibitory titres are largely influenced by :

(*a*) Slight increase in the temperature of incubation (White and Parker, 1938 ; Weld and Mitchell, 1939 ; White, 1939 ; Bratton, White and Marshall, 1939 ; Green, 1940).

(*b*) The size of the inoculum (Colebrook, Buttle and O'Meara, 1936 ; Wolff and Julius, 1939 ; Green, 1940).

(*c*) Variations in medium. It has been specially emphasized that streptococci can be killed when growing in serum alone, but that the effect is reversed or inhibited by the addition of peptone (Lockwood, 1938 ; McIntosh and Whitby, 1939). Weld and Mitchell (1939), however, point out that this is true for human serum but does not hold for rabbit serum, and is probably related to relative rates of growth in different sera and other less understood factors.

It seemed to the writers that some more light might be thrown on the

interesting possibilities suggested by Mayer's observations, if the relative activities of the products of oxidation of sulphanilamide were tested on a much wider range of bacteria, so that the connection if any between the respiratory mechanism of the bacteria and their sensitiveness to these compounds could be assessed. To carry through such an investigation by animal experimentation would obviously have involved an enormous amount of labour and expense, and it, therefore, seemed desirable to devise some cultural technique which would give a preliminary indication of the direction in which animal experiments might most profitably be made.

EXPERIMENTAL.

The theory of Mayer, stated briefly, is that it is an oxidation product of sulphanilamide intermediate between it and *p*-nitrobenzenesulphonamide to which bacteria are sensitive, in all probability *p*-hydroxylaminobenzenesulphonamide. If this assumption is correct, it should follow that bacteria like the pneumococcus and streptococcus, endowed in virtue of their peroxide-forming capacities with considerable oxidizing powers, should prove most sensitive to sulphanilamide under conditions in which their oxidizing activities were maximal, i.e. when cultivated on the surface of solid media.

The technique first investigated, therefore, was the preparation of plates of agar or heated blood agar in which various concentrations of the sulphanilamide compounds were incorporated, and the inoculation of these with suspensions of the bacteria to be investigated and dilutions of these.

As anticipated it was observed that, in such conditions, sulphanilamide developed considerable powers of restricting the growth of pneumococci, but very little capacity to arrest the growth of many other bacteria. Even the power to arrest pneumococcal growth varied much with the size and source of the inoculum. In view of these results we embarked on a systematic study of the relative bactericidal and bacteriostatic powers of the amino-, the hydroxylamino- and the nitro-compounds for many different bacteria, in fluid media with limited access to air on the one hand, and on solid media with free surface-air exposure on the other. The results of these observations are presented in a series of tables and figures.

It is clear in view of the observations quoted above on the influence of exact temperature, size of inoculum, etc., on the bactericidal effect of the sulphanilamide drugs that observations on different bacteria made at different times can only provide relatively comparable results. It is not claimed, therefore, that in the observations to be recorded the bactericidal titre as determined for sulphanilamide versus the gonococcus on the one hand and *Bact. coli* on the other are strictly comparable but only relatively so. Our principal purpose was to reveal the relative efficiency of these drugs in their various stages of oxidation. In each experiment simultaneous observations were made on the amino-, the hydroxylamino- and the nitro-compounds with one or more bacteria using the same sample of medium, the same inoculum and incubation in the same incubator. We believe that for the purpose of this comparison the observations are reliable.

TABLE I.—*Inhibitory Titres of Sulphanilamide, Its Oxidation Products and Sulphapyridine in Broth or Modified Broth.*

Bacteria.	Drug dilution found to produce total inhibition of growth.			
	Sulphanilamide.	Sulphapyridine.	Hydroxylamino-compound.	Nitro-compound.
Meningococcus	..	700,000	..	..
C. diphtheriae *mitis*	25,000	..	25,000	<2,000
C. diphtheriae *gravis*	12,500	..	14,000	1,500
Pneumococcus	500–10,000	1,000–>10,000	500–10,000	<500–1,000
Streptococcus	<500	..	500	800
Staphylococcus	<500	<500	<500	750
Cl. welchii	<500	<500	3000	50,000
Cl. novyi	<500	750	1500	40,000
Cl. septique	<500	1,500–5,000 (irregular)	750	50,000
Cl. sporogenes	<500	<500	<500	20,000
B. subtilis	<500	..	<500	1,500
Bact. coli	<500	<500	<500	500
Bact. typhosum	<500	..	<500	<500*
Bact. paratyphosum B	<500	<500	<500	1,000
Bact. shigae	<500	..	750	9,000
Bact. flexneri	<500	..	<500	3,000
Ps. pyocanea	<500	..	<500	<500
V. cholerae	<500	..	10,000	10,000

In this and the following tables the sign < means that a lower dilution than the one mentioned is required to inhibit the growth of the organism, and the sign > means that the highest dilution examined was completely inhibitory.

* There was considerable although incomplete inhibition with the nitro-compound at a dilution of 1/500, partial inhibition with the hydroxyl-amino-compound and no inhibition with the sulphanilamide at this dilution.

TABLE II.—*Inhibitory Titres of Sulphanilamide, Its Oxidation Products and Sulphapyridine in Agar ; Surface Culture.*

Bacteria.	Drug dilution found to produce total inhibition of growth.			
	Sulphanilamide.	Sulphapyridine.	Hydroxylamino-compound.	Nitro-compound.
C. diphtheriae *gravis*	30,000	40,000	10,000	1,500
C. diphtheriae *mitis*	30,000	30,000	35,000	1,000
C. diphtheriae *intermedius*	30,000	40,000	35,000	1,000
Pf. mallei	<1,000	..	>10,000	10,000
Pneumococcus	2,000–>10,000	..	1,500–15,000	3,000
Staphylococcus	<500	<500	4,500	1,200
Streptococcus	<500	<500	4,000	1,000
Str. faecalis	<750	<750	3,500	650
Bact. typhosum	<1,000	..	4,000	7,500
Bact. shigae	<500	..	15,000	40,000
Bact. flexneri	<500	..	6,000	10,000
Bact. coli	<500	<500	2,000	2,000
Bact. paratyphosum B	<500	<500	2,000	15,000
Ps. pyocyanea	<500	..	4,000	500
B. subtilis	<500	..	1,000	1,500
B. anthracis	<1,000	..	4,000	1,500
Br. abortus	<1,000*	..	3,000	6,000
V. cholerae	<500	..	3,500	50,000

For meaning of signs < and > see footnote to Table I.

* A smaller inoculum was inhibited completely even at a higher dilution.

Preparation of Oxidation Products of p-*aminobenzenesulphonamide.*

p-*Nitrobenzenesulphonamide.*

A mixture of *p*-nitrobenzenesulphonyl chloride (25 g.) and powdered ammonium carbonate (50 g.) was heated on the steam-bath for 20 min. ; the mixture was constantly stirred. The amide, crystallized from the minimum amount of boiling water, melted at 180° C. in agreement with the value recorded in the literature ; the yield was 20 g.

p-*Hydroxylaminobenzenesulphonamide.*

p-Nitrobenzenesulphonamide (9·6 g.) was reduced by the gradual addition of zinc dust (14 g.) to its solution in boiling alcohol (50 c.c.) containing water (12·5 c.c.) and ammonium chloride (1 g.) ; the mixture was shaken vigorously for one minute after each addition of zinc dust. The mixture was then filtered and the residual zinc dust washed with boiling alcohol (50 c.c.). The combined filtrates were diluted with water (50 c.c.), freed from alcohol by rapid distillation, and the aqueous residue then filtered from a small amount of insoluble material. The resulting, nearly colourless, aqueous solution deposited clusters of very fine needles when cooled in ice. The hydroxylamine, recrystallized from water, melted at 163–164° C. with decomposition (Found : C, 38·5 ; H, 4·5 ; N, 15·3 per cent. $C_6H_8O_3H_2S$ requires C, 38·3 ; H, 4·25 ; N, 14·9 per cent.) ; the yield was approximately 6 g. The compound reduces ammoniacal silver nitrate in the cold. When its aqueous solution is boiled for a time, or kept at room temperature especially in presence of air, crystalline material is deposited ; this is undoubtedly azoxybenzene—4 : 4^1—disulphonamide which does not melt up to 280° C. When aqueous solutions are kept in sealed tubes, little conversion into the azoxybenzene derivative occurs.

2-(p-*Nitrobenzenesulphonamido*)*pyridine.*

A mixture of 2-aminopyridine (5 g.), *p*-nitrobenzenesulphonylchloride (11 g.) and pyridine (50 c.c.) was heated on the steam bath for one hour. Dilution with water gave a solid which crystallized from glacial acetic acid in pale yellow needles decomposing at 175° C. (Found on material dried at 120° C. : C, 47·9 ; H, 3·2 ; N, 15·2 per cent. $C_{11}H_9O_4N_3S$ requires C, 47·3 ; H, 3·2 ; N, 15·05 per cent.) Yield 9·2 g. 2-(*p*-nitrobenzenesulphonamido)pyridine is very sparingly soluble in the usual organic solvents ; it can be recrystallized from pyridine.

2-(p-*Hydroxylaminobenzenesulphonamido*)*pyridine.*

A suspension of 2-(*p*-nitrobenzenesulphonamido) pyridine (6·5 g.) in a boiling mixture of alcohol (75 c.c.), water (6 c.c.) and ammonium chloride (0·5 g.) was treated with zinc dust (20 g.) added in portions of about 1·5 g. The mixture was then boiled for 40 min. and filtered from zinc dust and amorphous orange-red material formed during the reduction. The filtrate was diluted with water (50 c.c.), the alcohol removed by distillation, and the hot aqueous residue again filtered from coloured impurities. This aqueous filtrate, when cooled in ice, deposited 1·5 g. of a nearly colourless, microcrystalline solid,

which melted at 147° C. with decomposition after previous softening (Found : C, 49·4 ; H, 4·3 ; N, 15·9 per cent. $C_{11}H_{11}O_3N_3S$ requires C, 49·8 ; H, 4·15 ; N, 15·85 per cent.). Microscopic examination showed that the hydroxylamine was contaminated with a little of the above amorphous, coloured material. The compound reduced cold ammoniacal silver nitrate.

Effect of Sulphanilamide Compounds on Growth.

Observations in Fluid Media.

These were carried out in broth containing various dilutions of the sulphonamide derivatives in cases in which the bacteria under investigation grew freely in broth. Serum broth and broth reinforced with small amounts of heated blood were used, however, for bacteria like the pneumococcus and meningococcus incapable of growing freely in broth.

The list of bacteria investigated and their relative sensitivity to the sulphonamide compounds are shown in Table I.

The chief point that emerges from this table is that apart from meningococcus, which will be discussed more fully in connection with results on solid media, the only bacteria showing any considerable sensitivity to sulphanilamide or sulphapyridine in fluid media are the diphtheria bacillus and the pneumococcus. The effect with the latter, however, is very variable, being influenced greatly by the extent to which air has access to the culture. The discrepancy between the observations on diphtheria and those of Britton (1938) is most probably due to the very large inoculum which he used.

When the *p*-hydroxylaminobenzenesulphonamide is substituted for sulphanilamide, however, some additional varieties of bacteria exhibit sensitivity, notably *V. cholerae* and the anaerobes and, to a less extent, the Shiga dysentery bacillus and the streptococcus—all of them devoid of catalase or (*V. cholerae*) with very weak catalase activity. When, however, the nitro-compound is used there is a general and sharp rise in sensitivity, particularly with the anaerobes and the dysentery strains, but a relative diminution of sensitivity is recorded with diphtheria and pneumococcus, of which the growth had been most easily inhibited by sulphanilamide.

Observations in Broth Agar.

The sulphanilamide compounds were incorporated in the medium and the test organism inoculated on the surface. The figures obtained are shown in Table II.

As far as sulphanilamide and sulphapyridine are concerned the results are very similar to those obtained in broth. It is under these conditions, however, that the bactericidal and bacteriostatic properties of the *p*-hydroxylaminobenzenesulphonamide are most marked, exceeding as they do in the case of staphylococcus, streptococcus, *Ps. pyocyanea* and *B. anthracis* both those of the more reduced and the more oxidized stages of the same compound.

The majority of the bacteria tested, however, again show themselves most sensitive to the nitro-compound, and this is particularly true of the pathogenic forms which attack the body by way of the alimentary tract.

Observations in Agar Media Containing Blood or Serum.

Heated blood-agar.—Most tests were carried out in heated blood agar and the results are recorded in Table III. This medium, being well adapted to the cultivation of the *Neisseria* group, shows very clearly the remarkable sensitivity of bacteria of this group to these compounds. The cultural results show a number of close analogies with those obtained clinically, particularly in respect of the higher effectiveness of sulphapyridine. It is also possible to observe the relatively high sensitivity of *H. influenzae* to *p*-nitrobenzene-sulphonamide in this medium.

At first, when working with a stock strain of meningococcus and recently isolated strains of gonococcus, it seemed that the latter were so much more sensitive to these substances than the meningococcus that there was a wide discrepancy between the clinical observations and experiments *in vitro*. It was found, however, that when later on many recently isolated strains of meningococcus from the cerebro-spinal fluid were available, these were often fully as sensitive as the gonococcus to the action of sulphanilamide and its derivatives, and that in this respect also the method of surface culture in media containing the sulphanilamides yielded results closely resembling those obtained in the

TABLE III.—*Inhibitory Titres of Sulphanilamide, Its Oxidation Products and Sulphapyridine in Heated Blood Agar; Surface Culture.*

Bacteria.	Drug dilution found to produce total inhibition of growth.			
	Sulphanilamide.	Sulphapyridine.	Hydroxylamino-compound.	Nitro-compound.
Meningococcus { sensitive	40,000–500,000	400,000–4,000,000	15,000–40,000	50,000–600,000
Meningococcus { insensitive	2,500–5,000	10,000–70,000	4,000–8,000	10,000–30,000
N. catarrhalis	2,000	<10,000	7,500	15,000
Gonococcus	10,000–400,000	1,000,000–2,000,000	40,000	600,000
H. influenzae	<1,000	<1,000	2,500	40,000
C. diphtheriae { *gravis*	6,000	..	6,000	1,500
C. diphtheriae { *mitis*	..	..	..	1,100
C. diphtheriae { *intermedius*	..	..	..	1,100
Pneumococcus { sensitive	20,000–40,000	10,000–125,000	6,000–10,000	500–1,500
Pneumococcus { insensitive	1,000–5,000	1,500–10,000	500–2,000	500–1,500
Staphylococcus	<500	<1,400	750	800
Streptococcus	<500	<1,000	<1,000	750
Str. faecalis	<1,400	<1,400	..	..
Bact. typhosum	<500	..	<500	2,000
Bact. shigae	<500	..	<500	5,000
Bact. flexneri	<1,000	..	<1,000	3,000
Bact. coli	<500	<2,500	..	2,000
Bact. paratyphosum B	<500	..	<500	5,000
Br. abortus	10,000*	10,000*	irreg.	4,500†
Br. melitensis	<500	<500	<500	7,500
Past. pestis	4,500	5,000	<500	1,500
V. cholerae	<500	..	1,000	8,000
Cl. welchii	<500	<500	3,500	50,000
Cl. septique	<500	<500	1,500	20,000
Cl. novyi	<500	<500	5,000	40,000
Cl. sporogenes	<500	<500	3,500	20,000

For meaning of signs < and > see footnote to Table I.
* Fine growth from large inoculum down to 500.
† Complete.

human body. Another outstanding feature of the observations on blood agar is that this medium has a counteracting effect on the bactericidal and bacteriostatic action of the hydroxylamino-compound. Hydroxylamines are known to react rapidly with haemoglobin and its derivatives, and in this way it is probable that a good deal of the substance is removed from the field of action before the medium has been inoculated with bacteria.

Fresh blood agar and serum agar.—These were limited to the pneumococcus, streptococcus, staphylococcus and meningococcus, and in so far as explored did not diverge seriously from those made on " chocolate " agar apart from some colour effects which will be discussed in a separate publication.

Grouping of Bacteria in Respect of Their Relative Sensitivity to Sulphanilamide and the Products of Its Oxidation.

The bacteria tested can be roughly classified into five main groups. The character of these groups and the contrasts which they present are best expressed by figures in which the relative sensitivity of the bacteria to sulphanilamide, *p*-hydroxylaminobenzenesulphonamide and *p*-nitrobenzenesulphonamide respectively are indicated. These figures are based on the findings obtained for surface cultures on agar and, in the case of some bacteria, on

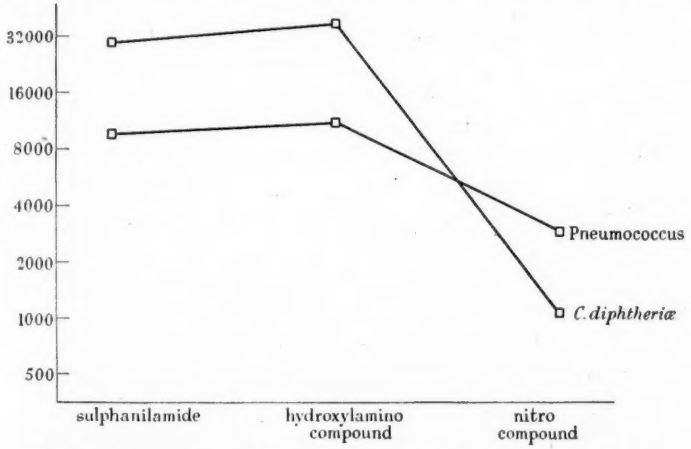

FIG. 1.—Diphtheria bacillus and pneumococcus on agar (Group 1, see text).

blood agar, since in those conditions the degree of air exposure of the culture is approximately constant—a result which is not obtained readily in fluid cultures. Considered in this way the bacteria investigated are resolved into the following five groups: (1) Those which are about equally sensitive to sulphanilamide and *p*-hydroxylaminobenzenesulphonamide but less so to the nitro-compound—the diphtheria bacillus and to a less extent the pneumococcus (Fig. 1). (2) Those which show a considerably higher sensitivity to *p*-hydroxylaminobenzenesulphonamide than to more reduced or more oxidized

substances from which it can be derived—the streptococcus, staphylococcus, *B. anthracis*, *Ps. pyocyanea* (Fig. 2). (3) Those which are remarkably sensitive to sulphanilamide but are still more so to the nitro-compound—the *Neisseria*

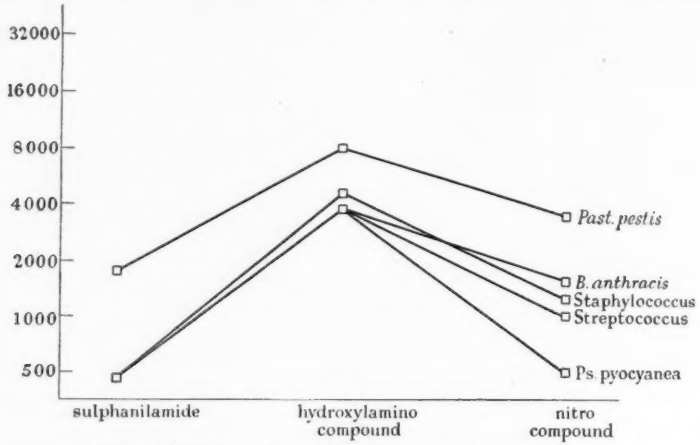

FIG. 2.—Streptococcus and other bacteria of Group 2 on agar.

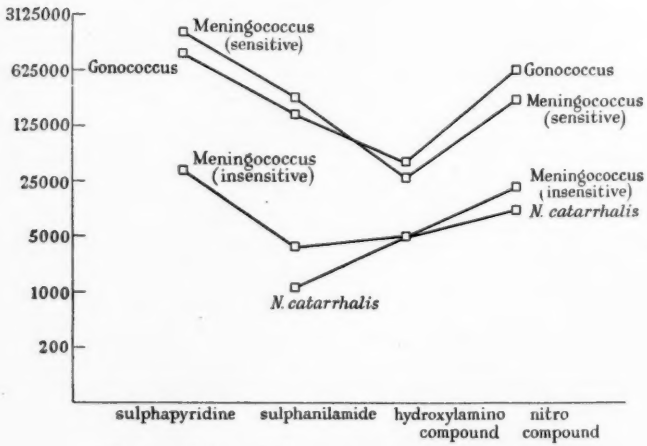

FIG. 3.—*Neisseriae* (Group 3) on heated blood agar.

group. As these bacteria are often difficult to grow if blood is excluded from the media, and, as blood has a counteracting effect on the activity of the *p*-hydroxylamino-compound, it is probable that the relatively lower titre shown for that compound in Fig. 3 is more apparent than real. (4) A group of which the genus *Bacterium* seems to be the most representative. These bacteria are

relatively insensitive to sulphanilamide and sulphapyridine, but are killed off by the *p*-hydroxylamino-compound more readily and are still more sensitive

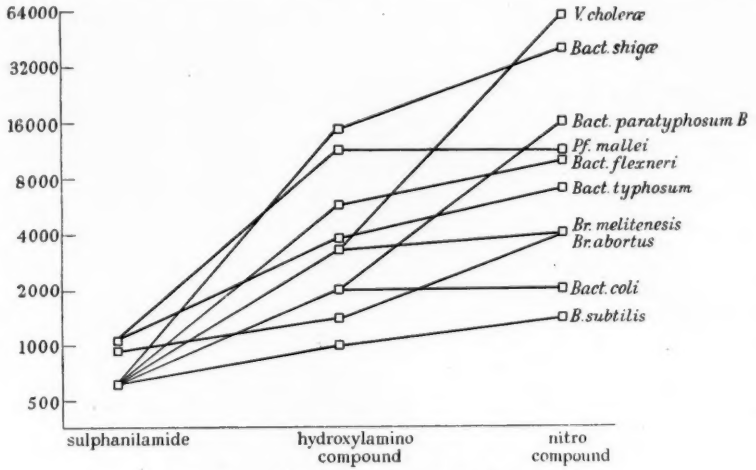

FIG. 4.—Bacteria of Group 4 on agar.

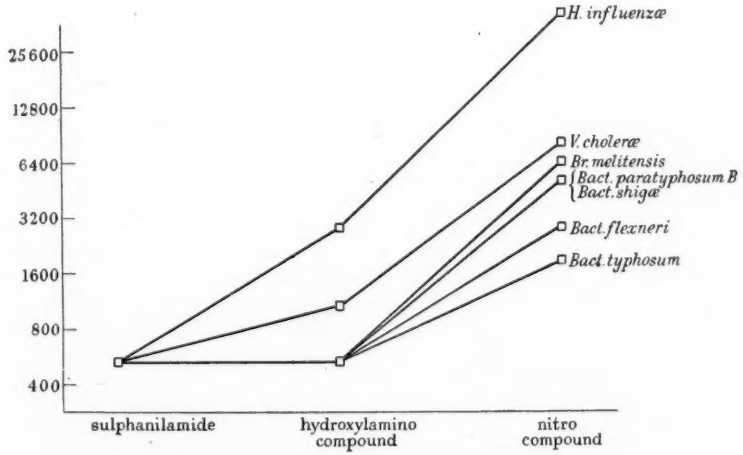

FIG. 4A.—Bacteria of Group 4 on heated blood agar.

to the *p*-nitro-compound (Fig. 4). It is interesting that the *H. influenzae* falls within this group with a specially noteworthy sensitivity to the *p*-nitrobenzenesulphonamide. The comparison between Fig. 4 and Fig. 4*a* demonstrates the marked influence of blood in suppressing the bactericidal effect of the

hydroxylamino-compound. (5) Lastly, the anaerobes are shown as a separate group, since they are biologically distinct and give very uniform results (Fig. 5). The chief difference between them and the foregoing group is that the rise in effective concentration on going from sulphanilamide through *p*-hydroxylaminobenzenesulphonamide to *p*-nitrobenzenesulphonamide is sharper and more regular.

In view of the possibility that the nitro-group as such might have an oxidizing effect which rendered it particularly toxic to the anaerobes, a number of organic nitro-compounds more or less related to the one under discussion was investigated. These included ammonium *p*-nitrobenzenesulphonate, an intermediate product used in obtaining *p*-nitrobenzenesulphonamide, and one

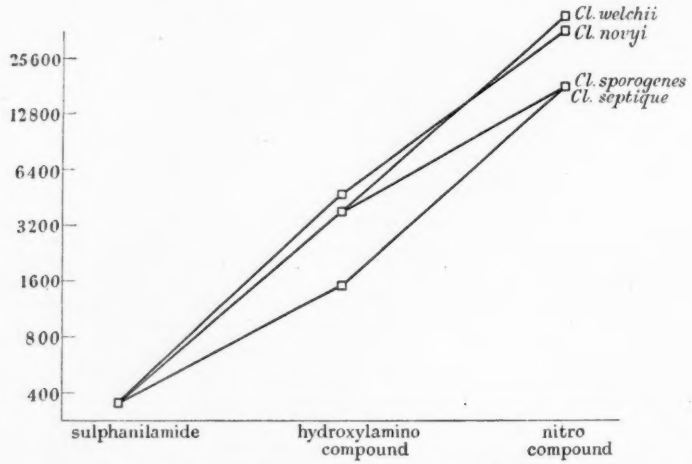

FIG. 5.—*Clostridium* group (Group 5) on heated blood agar.

very closely allied to it in constitution. The results of these investigations are given in Table IV, and show that the effect of the nitrobenzenesulphonamide on anaerobes is quite specific and not related to the organic nitro-group as such. It was also shown that the addition of *p*-nitrobenzenesulphonamide to broth did not especially raise its oxidation reduction potential; in fact it was inferior to sulphanilamide in this respect. Curves were obtained for us by Dr. K. I. Johnstone, using methods already described (Johnstone, 1940). It was found that in the presence of 1/5000 *p*-nitrobenzenesulphonamide, O.R. potential fell from + 0·29 to − 0·01 v. in nine hours at 37° C. under a current of nitrogen, the curve corresponding very closely to that obtained with broth alone; whereas, in the presence of a similar concentration of *p*-aminobenzenesulphonamide, the O.R. potential determined at the end of ten hours was still + 0·11 v. On substituting an air current for the nitrogen current all potentials returned in a few minutes to values approximating closely to + 0·27 v. This falls into line with the observation of Warren, Street and Stokinger (1939)

TABLE IV.—*Inhibitory Titre of Various Compounds for* Cl. Welchii.

Compound.	Medium. Broth.	Broth + 0·02 per cent. Na_2S.*	Heated blood agar.
Sulphanilamide [$NH_2.C_6H_4.SO_2NH_2$]	2000	<500	<500
p-Hydroxylaminobenzenesulphonamide [$NHOH.C_6H_4.SO_2NH_2$]	5000	5000	5000
p-Nitrobenzenesulphonamide [$NO_2.C_6H_4.SO_2NH_2$]	>50,000	50,000	50,000
Phenol [$C_6H_4.OH$]	5000 (irregular)	2000	1500
p-Nitrophenol [$NO_2.C_6H_4.OH$]	5000	2000	1500
p-Nitrobenzoic acid [$NO_2.C_6H_4.COOH$]	2000	2000	2000
Ammonium *p*-nitrobenzenesulphonate [$NO_2.C_6H_4.SO_3NH_4$]	<500	1000	<500
Controls (growth)	+ or −	+	+

* Freshly prepared solution.

that the oxidation reduction potential of broth is raised by sulphanilamide independently of the presence of bacteria.

DISCUSSION.

How far do these various observations support or tend to the rejection of Mayer's hypothesis that the *p*-hydroxylaminobenzenesulphonamide is the active substance ? In the first place his results with the *Streptococcus haemolyticus* are confirmed, although there are slight differences, e.g. the superiority of the hydroxylamino-compound is less marked in our observations and the nitro-compound is rather more active than the sulphanilamide.

The results with the diphtheria bacillus and the pneumococcus would also fall into line if it is supposed that these bacteria are capable of oxidizing the sulphanilamide but do not readily reduce the nitro-compound. This supposition is also favoured by the fact that light inoculations of pneumococcus indicate superiority of the sulphanilamide, whereas heavy inoculations, which would effect reduction more easily, are at least as sensitive to the nitro-compound,

TABLE V.—*Record of Growth after* 48 *Hours' Incubation of Agar Plates containing Different Concentrations of* p-*Amino- and* p-*Nitrobenzenesulphonamide. Surface Inoculated with Heavy and Dilute Suspensions of the Bacteria Indicated.*

Inoculum.	Drug.	Concentrations. 1–500.	1–1,000	1–2,000.	1–5,000	1–10,000.
Br. abortus Heavy inoculation	*p*-aminobenzenesulphonamide	0	+ (very fine)	0	+ (very fine)	+ (very fine)
Br. abortus Dilute „	*p*-aminobenzenesulphonamide	0	0	0	0	0
Br. abortus Heavy inoculation	*p*-nitrobenzenesulphonamide	0	0	0	+ (fine)	As control
Br. abortus Dilute „	*p*-nitrobenzenesulphonamide	0	0	0	0	„
Br. melitensis Heavy inoculation	*p*-aminobenzenesulphonamide	+ (fine)	++ (fine)	++	++	++
Br. melitensis Dilute „	*p*-aminobenzenesulphonamide	0	0	0	0	0
Br. melitensis Heavy inoculation	*p*-nitrobenzene sulphonamide	0	0	0	+ (fine)	As control
Br. melitensis Dilute „	*p*-nitrobenzene sulphonamide	0	0	0	0	„

the results being obtained only with greater concentrations of both drugs. It is an observation in any case with a number of bacteria that heavy inoculations may only be inhibited in their growth with the strongest concentrations of sulphanilamide used (1/500), whereas light inoculations are killed off at dilutions of 1/10,000 or more. In contrast light and heavy inoculations usually cease growing in the presence of the nitro-compound at the same concentration or very nearly so. This is illustrated in the form of an actual experimental record for *Br. abortus* and *Br. melitensis* presented in Table V. The fact that with the *Neisseria* group a result is obtained almost the reverse of that seen with the streptococcus, although at much higher dilutions of the sulphanilamide derivatives, may be explained by the fact that with their special oxidase mechanism these bacteria have unusual capacity for oxidizing sulphanilamide and that, since the observations are made in media containing blood, the titre shown for *p*-hydroxylaminobenzenesulphonamide is unduly low owing to neutralization of that compound by the blood in the medium. Where serum agar is substituted for blood agar in such experiments with the meningococcus, a marked rise of the titre of the hydroxylamino-compound is recorded. There remains, however, unexplained the fact that, in ordinary agar media, bacteria like *Bact. paratyphosum B* and *Bact. flexneri* are more sensitive to the nitro-compound than to the hydroxylamino-compound. This it seems to us can only be brought into line with Mayer's hypothesis in one of three ways. The first supposition is that the hydroxylamino-compound although the active one is rapidly changed after addition to the medium, and is therefore to some extent changed before the bacteria are exposed to its action. For this suggestion there is some reasonable ground in view of the very rapid spontaneous oxidation of this substance to insoluble coloured products in solutions exposed to air, whereas the nitro-compound, being transformed to the hydroxylamino-compound gradually and only under the action of the bacteria, would lose less of its effect. The second possibility is, of course, that the really active body is some product intermediate between the hydroxylamino- and nitro-compounds. The figures, which are most difficult to explain on Mayer's hypothesis, are those for *H. influenzae* and the anaerobes, against which the nitro-derivative is much more effective than the hydroxylamino- one. If the latter is indeed the active agent something over 90 per cent. of it must be decomposed in the medium before it exerts its action on the bacteria. In that case it might be expected that sulphanilamide would show a marked superiority to its hydroxylamino-derivative in inhibiting growth of organisms possessing high oxidizing powers, e.g. the pneumococcus. In fact there are no organisms for which sulphanilamide is more effective than the hydroxylamino-compound, but there are organisms for which the nitro-compound is more effective than sulphanilamide. If, however, the active substance is an oxidation product of sulphanilamide intermediate between the hydroxylamino-compound and the nitro-compound, the figures obtained for the different groups of organisms are those to be expected.

Lastly, there is the possibility that the nitro-compound is the most toxic to some bacteria, the sulphanilamide to others and the hydroxylamino-compound to a third group. This last seems to us an improbable hypothesis. If this, however, is a true theory of the action of the sulphanilamides it should

offer some explanation of the superior potency of the pyridine benzene sulphonamide compound introduced by Messrs. May & Baker, which is so widely acknowledged. Our own experiments *in vitro*, as well as those of others, show that this substance does not depend entirely on its lesser toxicity for its greater bactericidal effects. To bring it into line with Mayer's theory, therefore, it will be necessary to assume that the presence of pyridine in the molecule in some way facilitates its oxidation by bacteria to a hydroxylamino-compound or that the hydroxylamino-compound so formed is a more potent one. Unfortunately both the nitro- and hydroxylamino-compounds prepared from sulphapyridine were so highly insoluble, and the latter was so easily oxidized to other products, that reliable comparisons of their bactericidal potencies with that of the original substance could not be made. It was clear, however, that the hydroxylamino-substance was possessed of considerable anti-bacterial activity.

CONCLUSIONS.

1. When bacteria are inoculated on to the surface of solid media containing graded dilutions of sulphanilamide, *p*-hydroxylaminobenzenesulphonamide and *p*-nitrobenzenesulphonamide respectively they are found to be divided into at least four groups :

(i) Those more sensitive to the amino- than the nitro-compound.

(ii) Those more sensitive to the hydroxylamino- than to either of the other compounds.

(iii) Those very highly sensitive to sulphanilamide and slightly more so to the nitro-compound.

(iv) Those much more sensitive to the nitro-compound than to either of the other substances.

2. These observations favour Mayer's assumption that *p*-hydroxylaminobenzenesulphonamide or more probably some oxidation product intermediate between it and nitrobenzenesulphonamide is the active body.

3. Since *p*-hydroxylaminobenzenesulphonamide can be shown to be more active than the amino-compound on bacteria devoid of catalase in catalase-free media, the linking of Mayer's theory to the destruction of catalase by the hydroxylamino-compound as suggested by Locke, Main, Mellon and Shinn is not justified. The accumulation of hydrogen peroxide under the influence of these drugs also suggested by these and other American writers is not, however, necessarily, excluded.

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APPRAISAL OF THERAPEUTIC AGENTS IN EXPERIMENTAL STAPHYLOCOCCAL INFECTION.

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A NUMBER of reports (Barlow and Homburger, 1939 ; Bliss and Long, 1939 ; Bliss and Ott, 1940 ; Browning, 1940 ; Buttle, 1937 ; De and Basu, 1938 ; Farrell, 1940 ; Feinstone *et al.*, 1938 ; Mellon *et al.*, 1937 ; Rake and McKee, 1940 ; Whitby, 1938) have recently appeared which deal with the chemotherapy of staphylococcal infection in mice, but because of the difficulties inherent in testing, only a broad hint as to therapeutic efficacy is gained. An excellent review of this problem is given by Browning (1940). Consideration of these reports leads to the conclusion that a major difficulty in testing is due to the essential characteristics of the staphylococcus, which cannot be placed wholly within the group of microbes which kill by invasion without demonstrable production of exotoxins, or within the group of those which kill by virtue of strong diffusible toxins without invasion. Rather, it possesses properties of both groups to a marked degree. Thus, in tests made in these laboratories some 600 strains of pathogenic staphylococci from human sources have been found to produce diffusible toxins *in vitro*. More than 40 of these have been

tested upon mice, and when more than about three average lethal doses of a suspension containing little or no demonstrable free toxin were injected, the mice died very uniformly in a few hours with symptoms suggesting that death was due to acute toxaemia and not primarily to invasion. On the other hand, when only one average lethal dose was injected death was delayed, and the invasion of tissues in the more susceptible animals suggests that toxin was not the sole factor concerned. As a further complication, it has been shown (Farrell and Kitching, 1940) that when such a threshold dose of culture is employed, an agent of no real therapeutic value may decide the issue in favour of survival.

There are three possible solutions of this problem. First, one may attempt the difficult task of preparing a standard lethal dose which will avoid on the one hand the injection of too many organisms with attendant deaths from pure toxaemia, and on the other the injection of too few with consequent difficulty in interpretation of results. Even in this alternative, it is questionable whether the toxin produced by two or three average lethal doses can be entirely ignored. Secondly, attempts may be made to enhance virulence by passage through mice with and without the use of mucin. Extensive trials of these devices with 15 strains of staphylococcus have met with little success in our hands and were therefore abandoned. Our data are in accord with those reported by Browning (1940). Finally, one may neutralize the toxins with specific antitoxin, and for this purpose concentrated antitoxin as prepared for human use may be employed. This material, given in a prophylactic dose to mice, permits the injection of a sufficient number of microbes while preventing the toxic manifestations of infection and does not, so far as it is known, contain antibacterial antibodies. Further evidence on this point will be given here.

The following report deals with the development of this method of appraisal of therapeutic agents in pyogenic as distinct from toxigenic staphylococcal infection, and illustrates it by the testing of several members of the sulphonamide group of chemotherapeutic substances.

MATERIAL AND METHODS.

Lethal Test Suspension.

The strains used were " Wood 46," " 24 m.a.," isolated locally from an infected finger, and " Mouse 188," isolated locally from an abscess on the tail of a normal mouse. The first two are highly toxigenic, the *Lh* dose being 0·022 and 0·016 c.c. respectively. To minimize the content of exotoxins, the overnight growth of these two strains on agar slopes was washed off with broth and suspended in broth to a density corresponding with a standard suspension containing 4×10^8 cocci per c.c. and injected at once. Plate counts were always made and generally did not differ significantly from the standard. Sterile filtrates obtained one hour after suspension of the cocci may, to a dilution of 1/80, haemolyse 1 per cent. rabbit cells, but 1 c.c. has never been found to kill mice. An intraperitoneal injection of 0·5 c.c. of such a suspension containing 2×10^8 cocci causes death of mice in 4 to 5 hours and proves to be 5 average lethal doses (5 A.L.D.). Strain " Mouse 188 " (*Lh* dose = 0·06

c.c.) was used in only two tests reported here. An intraperitoneal injection of 0·3 c.c. of an overnight broth culture containing $2{\cdot}25 \times 10^8$ cocci was used and proved to be 2–3 A.L.D.

Control Mice.

At least 30 control animals were used for each test, 10 of which received the standard test dose given to the treated mice and died regularly in 4 to 5 hours. At least 5 received 0·5 c.c. of the standard suspension diluted 1 : 3, and usually all of these died in 6 to 8 hours. Ten received the standard suspension diluted 1 : 5. Theoretically this should be 1 A.L.D. and the death of these mice was irregular, half usually dying in 1 to 3 days and half surviving throughout. At least 5 received the standard suspension diluted 1 : 8, and usually all of these survived. This method is described in detail elsewhere (Farrell and Kitching, 1940).

Antitoxin Administration.

Three lots of horse serum concentrated for human use were used in varying amounts. Lots 53 and 55 were concentrated with ammonium sulphate and alum and contained 850 S.U./c.c. and 570 S.U./c.c. respectively. Lot 58 was concentrated by peptic digestion and contained 1300 S.U./c.c. For prophylaxis, one dose of antitoxin was injected subcutaneously 18–20 hours before the injection of living culture, since this route and interval had been found (Farrell and Kitching, 1940) most suitable. For treatment, antitoxin was injected subcutaneously each day for 5 days, the first dose being given immediately before the lethal test.

Drug Administration.

For most experiments the insoluble drugs were ground to a fine powder, suspended in 10 per cent. acacia and administered *per os*. In several preliminary tests, the first treatment was given immediately before or immediately after the lethal test. Later it was found advisable to give one treatment 18–20 hours before infection and the second treatment one or two hours before. Subsequent treatments were then given about 5 hours after infection, twice daily on the 2 or 3 days following and then once daily for about 4 days more. For administration in the food (Ehrlich, 1891) " Purina Fox Chow " was ground fine, sulphapyridine mixed in 1 per cent. concentration, a dough made with water, rolled out thin and dried *in vacuo*. Mice treated with sulphapyridine cake were kept in individual cages ; feeding was begun 2 days before infection and continued as long as mice survived up to 7 days, the uneaten food being weighed and replaced with fresh cake twice daily.

RESULTS.

Protective Power of Concentrated Antitoxin Alone.

In order to test the possibility that concentrated antitoxin might contain substances affecting the invasive activity of the microbes, the effect of daily treatment with antitoxin was investigated.

Thirty mice received a prophylactic dose of 0·5 c.c. of antitoxin 53. Twenty hours later and daily for 5 days one group of 10 received a dose of 0·25 c.c. and another group of 10 a dose of 0·5 c.c. of this serum. These 30 mice together with 10 untreated animals were tested in the usual way with the standard suspension of culture 24 m.a. (2×10^8). The controls which received 1 : 3, 1 : 5 and 1 : 8 dilutions of this suspension showed the lethal test dose to be 5 A.L.D. The results of this experiment, which was terminated at 49 days, are shown in Fig. 1.

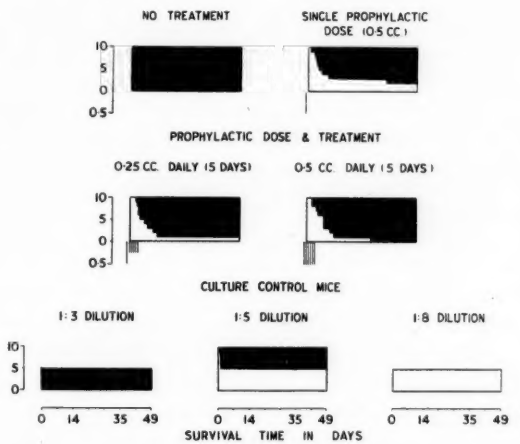

FIG. 1.—Effect of treatment with concentrated antitoxin only. Experiment terminated at 49 days. Ordinates above zero represent animals; below zero volume of serum (c.c.).

It will be seen that only 2 of the 10 mice which received the prophylactic dose of antitoxin survived for 49 days, and continued administration of antitoxin for 5 days did not increase the survival time. It seems clear, therefore, that the sole function of the antitoxin is to neutralize the toxin produced *in vivo* and that, provided an amount sufficient to accomplish this purpose is given in the beginning, further administration of antitoxin should not be expected to do more.

The possibility was investigated that batches of antitoxin concentrated according to different procedures might differ in protective power in relation to content of antitoxin. Three lots of antitoxin (53, 55 and 58) in volumes containing from 85 to 650 units of α antitoxin were given in a single prophylactic dose to groups of 8 or 9 mice, and all groups were tested with $1{\cdot}7 \times 10^8$ cocci of culture "24 m.a." In this test, which was terminated at 24 days, there was a wide variation in mean survival time and in the number of surviving animals. However, only two groups, the one which received 130 units of serum 58 and the one which received 85 units of serum 55, differed significantly ($\frac{\text{difference in mean survival time}}{\text{standard deviation of the difference}} = 3{\cdot}02$). The conclusion there-

fore appears warranted that no reliable increase in protection was obtained by increasing the prophylactic dose beyond about 100 units, and that these sera, concentrated by different processes, did not differ materially among themselves.

Treatment with Sulphapyridine Alone.

Five experiments were made to test the therapeutic efficacy of sulphapyridine alone. In one of these tests, 10 mg. of drug suspended in 10 per cent. acacia was injected subcutaneously immediately following infection with 5 A.L.D. of strain "Wood 46." In three tests 10 mg. was given *per os* in the same medium. In two of these three, the first dose was given respectively 1 hour and 2 hours before infection with approximately 5 A.L.D. of strain "24 m.a.," while in the other, two prophylactic doses were given, one 18 hours and one 3 hours before a similar infection. In the fifth test, sulphapyridine cake was fed to the mice for 2 days prior to infection with 2 A.L.D. of the less toxigenic strain "Mouse 188," and the average total consumption of drug during this period was $37{\cdot}9 \pm 1{\cdot}4$ mg.* So far as could be determined, the drug did not modify the lethal effect in any of these tests. For example, in the experiment in which the 10 treated mice received two doses of drug before the infection these mice died in $4{\cdot}54 \pm 0{\cdot}15$ hours and 10 untreated mice in $4{\cdot}63 \pm 0{\cdot}15$ hours. The other experiments gave similar results.

Combined Action of Antitoxin and Sulphapyridine.

The foregoing typical examples are sufficient to show clearly that neither antitoxin nor sulphapyridine alone protected mice against death from 5 A.L.D. of these strains of staphylococci. Experiments were therefore initiated to test the effect of serum and drug together. Sulphapyridine was tested in 4 separate experiments of which the following details and observations are typical. This method has also been used for testing 20 other compounds, and serves as a basis for trial of new chemotherapeutic agents in staphylococcal infections.

A prophylactic dose of antitoxin 53 was given to 20 mice and 10 of these received *per os* a dose of 10 mg. of sulphapyridine in 10 per cent. acacia. Eighteen hours later these 10 received a second dose of 10 mg. of drug. Two hours later all 20 mice together with 10 untreated animals received the lethal test of 2×10^8 cocci which the controls showed to be 5 A.L.D. Another dose of 10 mg. of sulphapyridine was given 5 hours later, then twice daily for 3 days and once a day for 2 days (11 doses). Autopsies were performed on all mice dying after injection of culture, and on all survivors when the experiment was terminated at 16 days.

The results of this experiment are shown graphically in Fig. 2.

As shown in Fig. 2, 9 of 10 mice which received only the prophylactic dose of antitoxin died in 16 days, while 8 of 10 which received treatment with sulphapyridine as well as prophylactic antitoxin survived throughout. Post-mortem examination showed one striking difference between the two groups. All but the sole survivor of the group which received antitoxin but no drug gave a positive culture from the lungs and most of them showed lesions in the lungs. On the other hand, only two of the group treated with sulpha-

* Standard deviation is used throughout this report as the measurement of variation.

pyridine gave positive lung culture. Other organs were essentially the same in both groups with many abscesses in liver, spleen and kidneys. These observations suggest that the untreated animals could have withstood gross infection of these organs, but developed a fatal pneumonia, while most mice treated with sulphapyridine survived because pneumonia either failed to develop or was overcome with the aid of the drug. That this second possibility is more probable was substantiated in a subsequent experiment in which 20 mice were treated with antitoxin and sulphapyridine and groups of 4 animals were killed 3, 7 and 10 days after infection. All mice killed at 3 days had lesions in the lungs and gave a positive lung culture, as did 1 killed at 7 days and 1 killed at 10 days. The lungs of all the 8 mice remaining after 43 days were

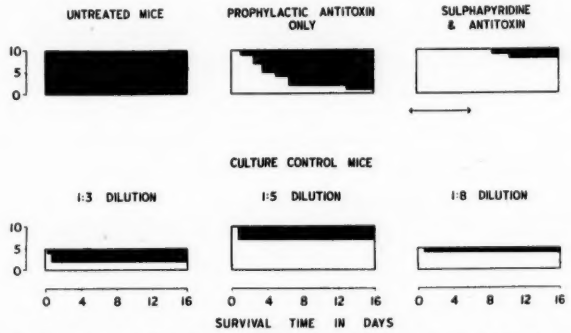

FIG. 2.—Effect of treatment with sulphapyridine in addition to prophylactic antitoxin (conc.). Experiment terminated at 16 days. Ordinates represent animals; ←→ sulphapyridine injections.

apparently normal. Other organs, as in previous experiments, resembled those in the group of mice untreated with drug.

Administration in the Food.

A logical way of treating mice with chemotherapeutic agents is that of mixing the drug with the food supply—a method introduced by Ehrlich (1891) and used by several workers recently. Although treatment with sulphapyridine in cake unaccompanied by antitoxin administration had not been successful, this method was also tested in combination with prophylactic antitoxin.

In the two days before infection, 10 mice consumed food containing $31{\cdot}7 \pm 0{\cdot}8$ mg. of drug. Nineteen hours before infection these 10 mice, together with 10 on a normal diet, received 0·3 c.c. of antitoxin 53. The usual control mice showed that the dose of $1{\cdot}4 \times 10^8$ of culture " 24 m.a." was not more than 3 A.L.D. During the first 9 hours after infection the 10 mice which were offered sulphapyridine cake consumed $6{\cdot}7 \pm 1{\cdot}4$ mg. of drug and only 2 of these, each of which received 14 mg. of drug, appeared lively. Twelve hours later each of these 2 had consumed about 18 mg. more of the drug, while the others had almost ceased to eat. Twenty-six hours after infection only these two were living and one died on the sixth day. Three of the 10 mice which

received only prophylactic antitoxin survived until the experiment was terminated at 24 days.

These results suggest that even in this less rigorous test (about 3 A.L.D.) the mice rapidly became too ill to consume enough drug to modify the infection, and that treatment by stomach tube is better in infections due to these strains.

Antitoxin and Thiazol Derivatives of Sulphanilamide.

Reports that sulphathiazol, sulphamethylthiazol and sulphaphenylthiazol were effective in the treatment of staphylococcal infections (Barlow and Homburger, 1939; Bliss and Ott, 1940; Rake and McKee, 1940) prompted the trial of these substances in combination with antitoxin. The technique of testing was as described for sulphapyridine and antitoxin. In a preliminary test in which these drugs and sulphapyridine were administered in doses of 40 mg., only sulphamethylthiazol gave significantly more protection than antitoxin alone, while the protection was significantly decreased with sulphaphenylthiazol. The probability was therefore that 40 mg. was too large a dose—a conclusion borne out by experiments in which varying doses of drugs were used. An analysis of the results is presented in Table I.

TABLE I.—*Treatment with Different Doses of Sulphathiazol and Sulphamethylthiazol.*

Experiment.	Groups.	Dose of drug *per os* in 10% acacia.	Mean survival time (days).	Groups compared.	D/S.D.*
A, terminated at 30 days	1	Sulphapyridine 10 mg.	$18{\cdot}8 \pm 4{\cdot}2$	1 with 2	1·4
	2	Sulphamethylthiazol 10 mg.	$11{\cdot}5 \pm 3{\cdot}1$	2 „ 3	2·4
	3	„ 20 mg.	$23{\cdot}3 \pm 3{\cdot}3$	3 „ 4	2·8
	4	„ 40 mg.	$12{\cdot}1 \pm 2{\cdot}2$	.. ..	..
B, terminated at 41 days	5	Sulphapyridine 20 mg.	$34{\cdot}8 \pm 4{\cdot}0$	5 „ 6	1·1
	6	Sulphathiazol 20 mg.	$27{\cdot}1 \pm 5{\cdot}5$	6 „ 7	1·7
	7	„ 40 mg.	$14{\cdot}2 \pm 5{\cdot}5$	5 „ 7	3·0

* $\text{D/S.D.} = \dfrac{\text{Difference in mean survival time of 2 groups}}{\text{Standard deviation of the difference.}}$

It will be seen that the survival time of the group treated with doses of 20 mg. of sulphamethylthiazol was significantly greater than that of the group treated with 40 mg., but 20 mg. of sulphathiazol was not significantly better than 40 mg. However, since 20 mg. of sulphapyridine gave better protection than 40 mg. of sulphathiazol (though not better than 20 mg.) it seemed evident that 20 mg. of these drugs was a reasonable dose.

When these three drugs were compared in doses of 20 mg. they were equally effective. The mean survival time of the group treated with sulphapyridine ($35{\cdot}5 \pm 6{\cdot}1$ days) was greatest and 7 of 10 mice survived throughout, but the difference from the group treated with sulphamethylthiazol ($29{\cdot}7 \pm 5{\cdot}9$ days) where 5 survived, or the group treated with sulphathiazol ($17{\cdot}8 \pm 6{\cdot}4$) where 3 survived, is not statistically significant.

The fact that the toxicity of compounds is apparent in this method of appraisal was shown very well in a series of tests of certain new compounds. Not only were some of these compounds ineffective as therapeutic agents, but mice protected with antitoxin and treated with them had a shorter survival time than mice which received only the prophylactic antitoxin. In each case,

the dose of drug used in treatment was below the *dosis tolerata** and below or equal to the dose* given several successive times without untoward effects. Nevertheless, in one group of 10 mice receiving one of these drugs in addition to prophylactic serum, 9 died in less than 22 hours, and the tenth died in 3–4 days. In a group of 10 receiving another drug tested at the same time all died in less than 22 hours. The 10 mice which received only the prophylactic serum had a mean survival time to 8 days of $3{\cdot}6 \pm 0{\cdot}4$ days. No experimental data are available to show whether this is due to the toxic effect of the drug upon mice already weakened by the infection or to actual promotion of infection by interference with the natural defences, as by destruction of leucocytes for example.

DISCUSSION.

The first object in presenting this report is to emphasize the difficulties inherent in testing the efficacy of therapeutic agents in staphylococcal infection. These difficulties depend in part upon the dual nature of this micro-organism, which combines the toxigenic activities of such microbes as *Cl. tetani* and *C. diphtheriae* with many of the invasive activities of those such as *Strep. pneumoniae* and *Strep. pyogenes*. In the appraisal of therapeutic agents these two aspects of staphylococcal infection should be clearly differentiated, and neither of them ought to be ignored because therapeutic agents may have their effect upon either of them or upon both.

Besides the difficulties arising from this dual nature of the staphylococcus, there are others which are found commonly in tests with various micro-organisms. It should be unnecessary to emphasize these elementary points, but the literature shows that the following ones have been repeatedly ignored. First, the variation in susceptibility of mice is very great, and unless all the control animals die the value of the experiment is much weakened. Secondly, agents which protect against one dose of living culture may be quite ineffective when a larger one is used. In each experiment, therefore, the number of average lethal doses of culture administered to the treated animals should be established by injecting an adequate number of groups of control animals with different dilutions of the standard suspension. Moreover, for the same reason, figures for survival obtained at different times with necessarily different dosages of culture should not be added and percentages worked out on the totals. Finally, in testing the efficacy of a new chemotherapeutic agent, different doses of the drug should be employed because most of them are in some degree toxic for the experimental animals and obviously each will have its own optimum dose for therapy.

The second object in presenting this report is to suggest that, because clinical infection is a combination of both the toxigenic and the invasive activities of the staphylococcus, a combination of antitoxin and drugs would appear to offer the best hope of success in the treatment of this infection. Experimental data on mice already reported (De and Basu, 1938; Farrell, 1940) lend support to this view and further evidence is supplied by the data presented here. In addition, a few unreported clinical trials of combined treatment with antitoxin and sulphapyridine have been strikingly successful.

* I am indebted for the data on these tolerance tests to Dr. R. Schnitzer, who also prepared the compounds undergoing test.

Finally, the use of antitoxin to neutralize the toxin produced *in vivo* by the lethal test suspension permits the appraisal of the efficacy of drugs in the treatment of the true pyogenic infection.

SUMMARY.

1. Staphylococcus antitoxin as concentrated for human use was found to be effective in neutralizing the toxin produced *in vivo* by 5 A.L.D. of toxigenic strains of living culture. Substances affecting the invasive activity of the micro-organism were not found to be present. Samples of antitoxin concentrated by ammonium sulphate and alum and by peptic digestion were found to be equally effective.

2. Sulphapyridine alone did not modify the lethal effect of 5 A.L.D. of the highly toxigenic strains used in these tests.

3. Mice protected with antitoxin against the toxaemia developed by the test culture were enabled by treatment with sulphapyridine to overcome an otherwise fatal issue and survive in spite of the development of local abscesses in liver, spleen and kidneys.

4. The combination of prophylactic serum and drug treatment has been developed into a method of appraising chemotherapeutic efficacy in staphylococcal infections.

5. Sulphathiazol, sulphamethylthiazol and sulphapyridine were found to be equally effective chemotherapeutic agents under the experimental conditions.

I wish to acknowledge with thanks my indebtedness to Prof. Donald T. Fraser, Associate Director of the Connaught Laboratories, who suggested that this work should be undertaken and gave encouragement and advice throughout the investigation, to Dr. R. Schnitzer, Research Associate in the Connaught Laboratories, who made many helpful suggestions and prepared a number of new compounds for test, and to Dr. H. Cave, Medical Director, Canadian Division, Winthrop Chemical Company Incorporated, for a generous supply of the thiazol derivatives of sulphanilamide used in these experiments.

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THE TECHNIQUE OF THE COAGULASE TEST FOR STAPHYLOCOCCI.

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THE property possessed by some staphylococci of coagulating citrated plasma (the coagulase test) is widely recognized as being a useful indication that the strain of staphylococcus is pathogenic. It has been found that staphylococci isolated from boils, abscesses and other obvious staphylococcal lesions elaborate this coagulase during their growth, whereas, in general, staphylococci isolated from the skin and not from any obvious lesion do not possess coagulase. The basis of the test is the addition of the organism to the plasma of citrated, oxalated or heparinized blood, the suspension being then incubated. After a varying period, the plasma clots to form a clear jelly-like mass. The coagulum later shrinks to form a smaller and more opaque mass floating in serum. The present work was undertaken in an attempt to find the most convenient method for carrying out the test.

In the experiments to be described the method adopted was the addition of a quantity of a fluid culture or suspension of the staphylococcus to be tested to 0·5 ml. of citrated plasma in a small tube. A counted number of drops of the suspension or culture was added to the plasma with a capillary pipette, each drop measuring 0·025 to 0·03 ml. After mixing, the contents of the tube were examined periodically and the time of formation of a clot was noted.

EFFECT OF VARYING THE CONCENTRATION OF THE CITRATED PLASMA.

A series of dilutions of plasma was prepared from blood containing 0·5 per cent. sodium citrate, normal saline being used as the diluent. Five drops of a 24-hour digest meat broth culture of a pathogenic staphylococcus were then added to 0·5 ml. volumes of each dilution and incubated in a water-bath at 37° C. The result obtained is shown in Table I.

TABLE I.—*Effect on the Clotting Time of Varying the Concentration of the Citrated Plasma.*

	Dilution of plasma.								
	Undiluted.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
Time in mins. of clotting	25	25	25	25	25	27	38	105	overnight.

A clot was obtained in all dilutions up to 1/256. At 1/256 clotting occurred only after many hours. It was found that all dilutions up to and including 1/32 produced a clot in approximately the same time. In the higher concentrations of plasma (undiluted and 1/2) the clot was less readily visible, as only

a small proportion of the plasma clotted at first. The 1/4, 1/8 and 1/16 dilutions gave a clot in which the whole of the diluted plasma participated, resulting in the conversion of the contents of the tube into a clear jelly. The higher dilutions gave a fibrous clot which, on shaking, condensed to a small white mass, generally followed later by the clotting of the whole contents. In order to obtain a readily visible clot in the shortest time with economy of material it was found that the plasma could be diluted ten or twenty times. In the following experiments a plasma dilution of 1/10 was adopted.

EFFECT OF VARYING THE AMOUNT OF ORGANISM ADDED.

Quantities of 1, 5, 10 and 15 drops of a 24-hour broth culture of a number of different pathogenic staphylococci were added to 0·5 ml. portions of diluted (1/10) plasma as in the previous experiment and incubated.

A typical result was as follows: With one drop clotting occurred in 40 minutes, with five drops in 20 minutes, with ten drops in 25 minutes and with fifteen drops in 35 minutes.

Five drops of the culture (about 0·125 ml.) added to 0·5 ml. of plasma was thus found to be the optimum amount, and there was delay in clotting if the amount varied widely from this. This agrees with the observation of Gengou (1933), who found that four or five drops of a broth culture was the optimum amount to be added to twenty drops of oxalated plasma.

EFFECT OF VARYING THE PERIOD OF INCUBATION OF THE BROTH CULTURE.

Digest broth cultures of pathogenic staphylococci, tested after various periods of incubation at 37° C., were found to take a certain minimum time, apparently characteristic of the strain, to produce a clot in plasma. This minimum was not demonstrated by cultures of three hours' incubation, but after six hours' incubation, a culture usually produced a clot in the minimum time over a wide range of volumes of added organism. An overnight culture of all the strains tested produced a clot in the mimimum time in most cases only with the 5-drop preparation. Further incubation failed to reduce this minimum time of clotting.

A broth culture of a staphylococcus was found to retain its clotting power unimpaired for as long as a week if kept at room temperature. After three weeks the culture was found still to possess coagulase, but the clotting time was longer. Fisher (1936) states that some cultures remain active for three months, others lose the power in one month. This retention of the clotting power was also noted by Cruickshank (1937) and Gengou (1933).

EFFECT OF VARYING THE CULTURE MEDIUM.

Staphylococci growing in a variety of media produce coagulase. Neither glucose broth nor peptone water were found to be as favourable media as simple digest broth.

Coagulase can also be demonstrated using an agar culture of a pathogenic staphylococcus. A suspension in broth of an agar culture of a staphylococcus was prepared of approximately the same opacity as an overnight broth culture.

The suspension was tested in the same way as a broth culture, and it was found that clotting occurred after a minimum of thirty minutes (using either five or ten drops in 0·5 ml. of plasma), while the minimum time of clotting of a 24-hour broth culture of this organism was 20 minutes. Similar suspensions were prepared in normal saline, distilled water and peptone water and compared with suspensions in broth. Broth was found to be the most favourable medium for the suspension, the clotting time being usually increased when using saline, peptone water or distilled water suspensions.

It was found that large quantities of staphylococci removed from agar and transferred directly into plasma either failed entirely to produce a clot, or produced only a small clot after many hours.

EFFECT OF VARYING THE AMOUNT OF SODIUM CITRATE.

A quantity of blood was divided into a number of portions, and to each portion a known amount of sodium citrate and normal saline was added. In this way a series of samples of plasma was obtained varying in citrate content from 0·4 per cent. to 2·5 per cent. To 0·5 ml. volumes of each plasma, five drops of a 24-hour broth culture were added. After incubation it was found that over the whole range of citrate concentration (0·4 per cent. to 2·5 per cent.) the variation in clotting time was relatively small, and between 0·4 per cent. and 1·3 per cent. the difference was only five minutes. (The actual clotting times are shown in Table II.) Thus, variations in the citrate content do not seriously affect the results.

TABLE II.—*Effect on Clotting Time of Varying the Amount of Citrate in Plasma.*

	Percentage of sodium citrate added to blood.					
	0·4.	0·6.	0·9.	1·3.	1·9.	2·5.
Time in minutes of clotting	23	23	28	28	33	38

EFFECT OF VARYING THE SOURCE OF THE PLASMA.

Blood was taken from each of six persons and citrated to 0·5 per cent. After separation, the plasmas were tested and all clotted in 25 minutes, with the exception of one, which took 35 minutes. This latter plasma was from a person who had recently had a staphylococcal infection. Plasma from a person suffering from an acute staphylococcal infection was compared with normal plasma and showed again a slight increase in the time taken for a clot to form.

EFFECT OF VARYING THE AGE OF THE PLASMA USED.

Undiluted, citrated plasma kept for several days under sterile conditions does not show any marked variation in the time of clotting. Plasma which had been kept for three weeks at room temperature was diluted and compared with fresh plasma. The time of formation of a clot was practically the same, being 40 minutes in the case of the fresh and 45 minutes in the case of the stale plasma. Diluted plasma kept in a plugged tube for three weeks clotted much more slowly.

EFFECT OF VARYING THE TEMPERATURE AT WHICH THE EXPERIMENT IS CONDUCTED.

Volumes of 0·5 ml. of plasma were incubated with five drops of a broth culture of a staphylococcus at 37° C., 45° C., 56° C. and room temperature. Clotting was found to be quickest (25 minutes) at 37° C., at room temperature the clot formed in about twice the time (55–80 minutes), while the preparations at 45° C. and 56° C. failed to clot after sevéral hours.

CONCLUSION.

The method suggested for the performance of the coagulase test is as follows :

1. Dilute citrated human plasma 10-fold with normal saline.
2. Place 0·5 ml. of this diluted plasma in two small test-tubes.
3. To one of these add five drops from a capillary pipette (approximately 0·125 ml.) of an overnight broth culture of staphylococcus or a suspension of an agar culture made in broth to an opacity approximately equal to an overnight broth culture. The second tube serves as a control and must show no clotting. Incubate the tubes at 37° C.
4. Examine the tubes after half an hour and at intervals for six hours. (Clotting of the plasma usually occurs within one hour.)

SUMMARY.

1. Within wide limits the amount of sodium citrate does not materially affect the coagulase test.
2. Citrated plasma diluted 10 or 20 times gives satisfactory results.
3. Human plasma from different individuals does not show wide variations in clotting time.
4. The citrated plasma remains suitable for the test for several weeks if kept undiluted under sterile conditions.
5. Staphylococci growing in a variety of media produce coagulase. Of a number of liquid media, broth gave a culture which produced coagulation most quickly. Agar cultures may also be employed, in which case it is best to emulsify the organism in broth to form an even suspension of an opacity approximately equal to that of a 24-hour broth culture.
6. There is an optimum ratio between amount of organism and amount of plasma.
7. The reaction occurs most quickly at 37° C.

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